THE RESPONSE OF GUINEA PIG EPIDERMAL MELANOCYTES IN CULTURE TO NERVE GROWTH FACTOR

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by

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INTRODUCTION

Researchers have devoted much attention to the studying of melanocytes. The differences in colors and hues of human complexions are made possible by melanin, a protein material synthesized within specialized skin cells called melanocytes. In all races, the number of melanocytes is approximately the same but the various skin colors are the results of the differences in the amounts and distributions of melanin within the epidermal cells. However, at present, it is difficult to observe the dynamics of the interaction between melanocytes and keratinocytes *in vivo* and researchers have attempted to simplify their studies by observing these cells *in vitro*.

Growing melanocytes *in vitro* has become an important research tool for those interested in pigment cells. For examples, researchers have used melanocytes in culture to study pigment transfer (Cruickshank et al,1960; Cohen & Szabo,1968; Klaus, 1969; Prunieras,1969), and the toxicity of certain carcinogens (Klaus & Minerbi,1967; Serri et al,1973). The importance of growing melanocytes in culture for future research has been further emphasized by Klaus (1979). He suggested that by studying melanocytes in culture one could gain a better understanding of melanoma, a cancer of pigment producing cells. For example, one could study the transformation of normal melanocytes into melanoma cells. We can study, *in vitro*, the influence of viral, toxic, mutagens, genetic, and immunologic

factors on the transformation of melanocytes. Vitiligo is another disorder involving pigment cells that can benefit from studying melanocytes in vitro. Vitiligo is characterized by depigmented patch in the skin caused by the disappearance of pigment cells from the epidermis. With an improved technique for culturing melanocytes, it may be possible to harvest marlanocytes from individuals suffering from vitiligo and see if they are different from melanocytes from normal individuals.

TECHNIQUES FOR CULTURING MELANOCYTES

Unfortunately, attempts at culturing and growing melanocytes in vitro have not been very successful. Hu and colleagues (1957), using human skin explants, were able to grow human melanocytes in culture, but noted their disappearance from the culture after several weeks. Cruickshank et al (1960) and Prunieras (1967) reported some success in growing human melanocytes using a trypsinization technique. A modification of Cruickshank method combining EDTA, trypsin and mechanical disruption, has made it possible to culture a large numbers of human melanocytes (Kitano, 1970). At present, a reliable procedure for a long-termed culturing of human melanocytes has not yet been established. It is hoped that the knowledge gained from guinea pig melanocytes in culture will be used to develop such a method.

Ever since the introduction of Cruickshank method,

researchers have tried to culture melanocytes, freed from keratinocytes. This problem is made difficult by the fact that melanocytes, *in vivo*, do not exist in a homogeneous cluster, and that they are located both under and wedged between the epidermocytes (Drzewiecki & Kjaergaard, 1977). Furthermore, the turnover rate of melanocytes is quite low and they are soon overgrown by the more rapidly dividing keratinocytes.

Several methods to enhance culture of melanocytes have been tried, but most have been unsuccessful. For example, Riley (1970) reported that by incubating the melanocyte-keratinocyte suspension from black guinea pig in 95% oxygen and 5% carbon dioxide, he could obtain a pure culture of melanocytes. He attributed this to the fact that melanocytes were more resistant to higher oxygen tension than keratinocytes.

By treating the cell suspension for 3 minutes in a solution of 0.9% sodium chloride containing 0.8% sodium citrate, Prunieras et al (1976) could obtain almost a pure culture of melanocytes after 3-4 days in culture. However, the number of selected melanocytes was low, and approximately only 1 out of 40 melanocytes planted was recovered. The selectivity offered by sodium citrate treatment was thought to be due to the different requirements of Ca^{++} and Mg^{++} in cell attachment. Melanocytes can attach themselves to the glass coverslip, in the absence of Ca^{++} and/or Mg^{++}, whereas keratinocytes cannot.

In a similar fashion, Fritsch and his collaborators (1979) obtained almost pure cultures of melanocytes, making use of

the differential serum requirements of guinea pig melanocytes and keratinocytes. They discovered that, in contrast to melanocytes, keratinocytes could not attach themselves and grow if the media used contained guinea pig serum, instead of fetal calf serum. They were able to isolate a protein, keratinocyte attachment factor, from the fetal calf serum but not from the guinea pig serum.

Researchers have made use of the size differences during cell cycle to separate cells into the various phases of the cell cycle. It may be feasible to separate melanocytes from keratinocytes on the basis of size and density differential, using centrifugal elutrition. Any stimulus that can increase the cell size and the number of dendrites of melanocytes, without affecting keratinocytes, can be used to enhance cell separation by an elutriator.

NERVE GROWTH FACTOR AS AN AGENT FOR ENHANCING MELANOCYTE GROWTH

One approach that might prove fruitful is the addition of nerve growth factor (NGF) to the melanocyte-keratinocyte suspension. NGF is known to affect sympathetic ganglia and pheochromocytoma cells, both neural crest derivatives. It is reasonable to expect that NGF may have a stimulatory effect on melanocytes specifically, since they, too, are neural crest derivatives. Furthermore, it has been reported that there are NGF receptors on the cell membrane of human melanoma cells

(Fabricants et al,1977;1979; Sherwin et al,1979), and that mouse melanoma cells can survive in NGF supplemented, serum-free medium (Mather & Sato,1979). Thus, it appears that NGF has, at least, a " trophic " effect on mouse melanoma cells and, if we extrapolate the interpretation one step further, NGF ought to have some effects on melanocyte dendrites.

Nichols and colleagues (1977) performed an experiment demonstrating the effect of NGF on the maturation of melanocytes. From the spinal ganglia of chick embryo, they could recover melanocytes from the explants in vitro. One of the factors influencing melanogenesis was NGF activity in the culture medium. They reported that minimal medium without NGF activity could not support melanogenesis, and the medium that permitted melanogenesis had NGF activity. To explain this, we have to compare the melanocytes to sympathetic neurons. Both are originated from common precursors, the neural crest cells, and utilize the same raw material, tyrosine, for its secretory products, epinephrine and melanin, respectively. In the sympathetic neuron, the conversion of tyrosine to dopa requires an enzyme, tyrosine hydroxylase, whereas the melanocyte requires another enzyme, tyrosinase (Blois,1974). Since NGF has been shown to increase tyrosine hydroxylase activity (Thoenan et al,1971), it is conceivable that NGF may also stimulate tyrosinase activity.

Of particular interest to this experiment is the effects of NGF on tubulin and cAMP. Levi-Montalcini and Calissano (1979)

have reported that NGF enhances polymerization of tubulin and actin to give rise to microtubules and microfilaments respectively in neuronal cells. They hypothesised that the formation of microtubules and microfilaments, induced by NGF, might have led directly to the growth and elongation of the nerve fibers. Since microtubules and microfilaments are also components of the dendrites (Klaus & Suvanaprakorn,1973), it would not be surprising that the addition of NGF to melanocytes in culture could lead to an elongation and an increase in the number of dendrites.

NERVE GROWTH FACTOR

Nerve growth factor (NGF) is the generic name given to a group of proteins which produce hypertrophy and hyperplasia of the sympathetic and embryonic sensory neurons. It is also known for its ability to induce outgrowth of fibers from explants of embryonic chick sympathetic and sensory ganglia (Levi-Montalcini & Hamburger,1953).

History

The presence of NGF was first reported by Bueker in 1948 (Levi-Montalcini,1960). He transplanted fragments of mouse sarcoma 180 to the body wall of 3 day chick embryos and found that the fragments were heavily innervated by sensory fibers

from the adjacent spinal ganglia. Sensory and sympathetic ganglia close to the tumor were found to be hyperplastic and hypertrophic. The sympathetic ganglia that did not innervate the tumors were also found to be enlarged. The same effect could be reproduced when fragments of mouse sarcoma 180 were transplanted onto the chorioallantoic membrane of 4-5 day chick embryo. Furthermore, when explants of embryonic chick sympathetic ganglia were incubated in the cell-free homogenate of the tumors, dense halo of nerve fibers were observed surrounding the explants. It was concluded that a soluble tumor factor was responsible for the stimulatory effects on the nerve cells (Levi-Montalcini & Hamburger,1954).

In an effort to purify protein extracts from the tumor, snake venom was added to degrade nucleic acids. The final preparation resulted in a tremendous increase in growth promoting activity, more than was anticipated. Thus, the snake venom itself was tested for NGF activity and found to be a potent growth promoter (Cohen & Levi-Montalcini,1956). With a more abundant source, Cohen (1959) was able to isolate and purify a nerve growth promoting protein which he termed nerve growth factor (NGF).

Searching for a richer source of NGF, Cohen directed his attention to roden salivary glands. He reasoned that since snake venom was produced in a modified salivary glands, a logical place to look for would be the salivary glands. Subsequently, he succeeded in isolating an NGF from mouse submaxillary glands

that was 10,000 times more potent than the original mouse sarcoma extracts, and 10 times more active than the snake venom NGF (Cohen,1960). Approximately 0.3-5 mg of NGF could be harvested from one gram of mouse submaxillary glands (Harper et al,1979).

While salivary glands of rat, guinea pig, rabbit, cow, and human contained no detectible NGF activity, it was found to be widely distributed in mammals. Aside from mouse sarcoma, certain cell lines were reported to be NGF producers, for examples, rat adrenal medulla cells, rat C 6 glioma cells, neuroblastoma C1800 and human melanoma cells (Sherwin et al,1979). Sera of certain mammals, human included, were shown to have NGF activity (Table 1). NGF is not limited only to the salivary glands, other tissues are reported to contain NGF, though at a much lower concentration. For example, brain and superior cervical ganglia of male mouse contain a tiny fraction compared to the submaxillary glands (Greene,1977). A rich source of NGF, comparable to the mouse submaxillary glands, is the guinea pig prostate gland (Harper et al,1979). The significance of this finding is, at present, unclear.

Physical Properties

Two forms of NGF were isolated from mouse submaxillary glands. The 7 S NGF was isolated by Varon et al (1967) and the 2.5 S NGF was isolated by Bocchini and Angelletti (1969). It was subsequently demonstrated that the 2.5 S form was a

component of the 7 S NGF, and that biologically active fraction of the NGF was localized to the 2.5 S form. With large quantities of NGF made available by the method of Bocchini and Angelletti, Angelletti and Bradshaw were able to determine the amino acid sequence of the 2.5 S NGF (1971).

The 2.5 S NGF is a dimer of 2 identical subunits, a polypeptide of 118 amino acids and a molecular weight of 13,259 (Angelletti & Bradshaw,1971). Figure 1 is a schematic representation of the polypeptide subunit. The 2 subunits are held together by 3 disulfide bonds. Analysing its molecular structure, Frazier and colleagues (1972) reported the similarities of the hormone insulin to NGF in its primary and tertiary structures. Immunologic studies have shown mouse NGF to be different from NGF isolated from snake venom and guinea pig prostate gland, but they are closely related and have similar biological activities (Angelletti & Bradshaw,1971; Harper et al, 1979).

It is hypothesized that the storage form of NGF, the 7 S NGF, is a complex of 2.5 S NGF, 2 gamma, and 2 alpha subunits. The gamma subunits is an enzyme with arginine esteropeptidase activity. Two gamma subunits attach themselves to a proNGF polypeptide chain and cleave the proNGF to generate the active dimer, a 2.5 S NGF. Unlike other enzymes, the 2 gamma subunits remain attached to their end products. Together with 2 alpha subunits, they protect the 2.5 S subnit from further degradation by other enzymes , figure 2 (Mobley et al,1978).

NGF is heat labile, non-dialysable and hydrolysed by acid. It is stable to alkali and to 6 N urea (Levi-Montalcini,1966). It binds to proteins, particularly the alpha-2-macroglobulins in sera of several species (Ronne et al,1979). It is adhesive and adsorbs to glass, polypropylene, silicone, and to a lesser extent, to polystyrene and teflon (Pearce,et al, 1973). Loss of activity can be minimized by using polystyrene labware and diluting NGF in a protein solution.

Effects

In addition to its well known effects in embryonic chick sympathetic and sensory ganglia, researchers have found that other cells are also affected by the NGF. When injected into neonatal rats from birth to the 5th day with 1000 BU per gm of animal (1 BU, Biological Unit, is approximatedly 10 ng which is the amount needed per ml to induce a certain amount of fiber outgrowth from embryonic chick sympathetic ganglia in vitro), NGF induces the growth of neurons in the superior cervical ganglia to 1.3 times the diameter of the control. It appears that NGF does not stimulate mitosis but seems to increase the neuronal survival and accelerates the maturation of "stem cells" (Hendry, 1976; Hendry & Campbell,1976). Besides increasing cell size, NGF has been reported to induce the neurite outgrowth in intact axon of adult mice (Bjerre et al,1975), and in rat pheochromocytoma cells (Tischler & Greene,1975; Greene & Tischler,1976).

The effects of NGF in non-neuronal cells have been demonstrated by Radeva (1978). He incubated newt embryo in NGF containing medium and found that NGF induced maturation and hypertrophy of spongioblasts and myoblasts.

Metabolic effects of NGF have also been extensively studied. It enhances glucose utilization in the sympathetic ganglia by increasing hexose monophosphate shunt (Angelletti et al,1964b), increases the rate of acetate incorporation into lipids (Angelletti et al,1964a), stimulates amino acid uptake and incorporation into protein and increases RNA synthesis (Angelletti et al,1965). NGF also stimulates certain enzymes, including tyrosine hydroxylase (Thoenan et al,1971), and ornithine decarboxylase (Macdonnell,1977). In addition, NGF appears to serve as a "trophic" factor for certain cells in vitro. These cells cannot be maintained in serum-free media, but survive when NGF is added to the serum-free media. Examples of these are dissociated neurons of chick sympathetic and sensory ganglia (Levi-Montalcini & Angelletti,1963), rat pheochromocytoma cells PC 12 (Greene, 1978), and mouse melanoma cells (Mather & Sato, 1979).

Since recent studies have demonstrated the presence of NGF receptors on human melanoma cell and it has been postulated that NGF might have stimulatory effect on the melanoma cells, the purpose of this thesis is to explore the effect(s) of NGF, if any, on normal guinea pig melanocytes in vitro. The study will focus on dendritogenesis and cell size, primarily because these effects are seen in embryonic chick sensory and sympathetic neurons (Levi-Montalcini & Angelletti,1963), and in a rat pheochromocytoma cell line (Greene,1977). If NGF were shown to increase cell size and stimulate dendritogenesis, the information gained might enable us to improve the technique for growing melanocytes in vitro.

The dendrite outgrowth and cell expanding effect of NGF were compared to dibutyryl cyclic AMP and a control. Dendritogenesis and cell enlargement were assayed using tracings from camera lucida and phase contrast microphotography.

The concentrations of NGF were 10, 50 and 100 ng/ml. Greene (1974) showed that the maximum response in neurite outgrowth of dissociated chick embryonic sympathetic ganglia occured at 0.3-0.4 ng/ml and that of the clonal pheochromocytoma PC12 cell line was 1 ng/ml (Greene,1977). Hence, the concentrations used in this experiment were chosen to cover a wide range.

MATERIALS AND METHODS

Materials

Cell Source:

Epidermal cells were cultivated from adult black and white guinea pigs according to the method of Cruickshank et al (1960). Only skin from the dorsum of the ear was used.

Culture Media:

Eagle minimum essential medium with 2 mM l-glutamine was supplemented with 30% fetal calf serum. To control bacterial growth, gentamicin sulfate was added to make a final concentration of 100 ug/ml. The pH of the medium was kept at 7.2-7.5.

0.25% Trypsin:

Trypsin (Difco 1:250) was dissoved in Ca^{++} and Mg^{++} free Tyrode solution. Approximately 3-4 ml of fresh 0.25% trypsin was placed in a petri dish and kept in an incubator at 37°C for 30-45 minutes, prior to the culturing procedure.

NGF:

A stock solution of 1 ug/ml NGF was made by dissolving 10 ug of NGF (Collaborative Research) in 10 ml of Eagle MEM. To minimize loss of activity through adsorption, the solution was stored, in small aliquots, in polystyrene labware and kept frozen at -20°C until use. In solubilized form, NGF remains stable at -20°C for 1 month. The pH was kept at 7.2-7.8.

DBcAMP:

DBcAMP (M.W. 491.4) was dissolved in distilled water to make a stock solution of 20 mM. This was kept frozen at -20°C.

Theophylline:

20mM solution was made by dissolving theophylline (M.W. 180.2) in distilled water and storing at -20°C.

Methods

After the animal had been anesthetized with ether, one ear was cleansed with 70% alcohol and closely shaved to remove as much hair as possible from both surfaces of the ear. The dorsum of the ear was cleansed again with 70% alcohol, and shave biopsies were performed with a knife (#11 blade). The biopsies, measured approximately 3-5 mm in diameter, were then transferred to a pre-warmed solution of 0.25% trypsin at 37°C.

The skin fragments were floated dermal side down and incubated in trypsin solution at 37°C for 15-30 minutes. In general, the thicker the specimen, the longer it took for the enzyme to separate the dermis from the epidermis. The specimens were then transferred to a second petri dish containing 0.5 ml of culture medium at 37°C.

With a paired needles (# 19 guage), the dermis was slowly peeled from the epidermis. Melanocytes attached to the dermis were identifiable because of the dark coloration imparted on the dermis. These were removed by gently shaking the dermis in the

culture medium with the paired needles. The dermis was then discarded.

To remove melanocytes from the epidermis, the sheets were placed keratin side down and the cells from the basilar side of the grafts were dispersed into the medium by gently scraping with the paired needles. After all the grafts had been treated, debris from skin and hair fragments were removed from the medium containing cell suspension.

Clumps of cells were triturated by aspirating and gently forcing the cell suspension through a #20 guage needle into a clean petri dish. An aliquot of the cell suspension was removed and the cell concentration was determined with a hemacytometer. Medium was added to produce a suspension of approximately 300,000-500,000 cells/ml.

About 0.2 ml of the cell suspension was injected into a pre-warmed Cruickshank chamber and the aperture was sealed with beeswax. The chambers were incubated coverslip-sides down in a moisturized incubator at 37°C for a period of 24 hours to allow the cells to adhere to the underside of the coverslips. After 24 hours the chambers were righted, and the media with unattached cells were removed with hypodermic needles. It is unlikely for cells that do not attach within 24 hours to become attached to the coverslip, upon further incubation (Prunieras,1976). Fresh medium was injected into the chamber and the aperture was resealed with beeswax. The chambers were incubated right side

up and media were, thereafter, changed 3 times a week.

After the cultures had been established, the keratinocyte-melanocyte mixture was exposed to either NGF or DBcAMP-Theophylline. The control group was maintained in MEM supplemented with 30% fetal calf serum. The ages of the cultures, prior to the addition of NGF or DBcAMP-Theophylline, ranged from 4-7 days. The day that NGF or DBcAMP-Theophylline was added was designated as Day 0.

In the NGF treated group, the cell cultures were exposed to either 10, 50, or 100 ng/ml of NGF. A small aliquot of frozen 1 ug/ml NGF was thawed and diluted with the culture medium to the appropriate concentrations of 10, 50, and 100 ng/ml. The protein in the medium minimized loss of activity from adsorption to the glass chambers. Medium change was carried out three times weekly with a newly prepared medium containing appropriate concentration of NGF. The cell cultures were incubated in NGF for periods of 5-10 days.

The second group of cells was incubated in media containing 0.5 mM DBcAMP and 0.5 mM theophylline. Fresh solution were used for each medium change.

The cells were observed under phase contrast microscopy. Changes in shape and size of the melanocytes were documented by photography (Kodak Ektachrome 400), and tracing with camera lucida. At each observation, the melanocytes from each chamber were categorized into 5 different morphological groups, according to the number of dendrites. Since there was no

clear cut boundary existed between cell body and dendrite, it was necessary to establish a criterion for dendrite. In this experiment, a dendrite was arbitarily defined as any cellular process whose length was at least one fourth of the diameter of the cell body. On this basis, the cells were divided into: a) rounded - those without any dendrite, b) 1 dendrite, c) 2 dendrites, d) 3 dendrites, and e) more than three dendrites. See figure 3.

For determination of cell size, the surface areas of melanocytes were traced on paper, using camera lucida. For each group, 10 cells were drawn, at random, on the same day. The surface area of each tracing was determined by a compensating polar planimeter (Keuffel and Esser 620015).

RESULTS

Early Appearances:

After 24 hours of incubation, the cultures of epidermal cells appeared fairly uniform. Many cells had attached to the coverslips and some appeared flattened. Those that did not survive remained round and appeared highly refractile. They were washed away, with unattached cells, during the medium change. At this stage, melanocytes could be distinguished from keratinocytes by the amount of pigment granules within the cells. The melanocytes appeared dark brown, while the keratinocytes were greyish white in color.

After 2-3 days, the keratinocytes became larger in size and appeared spread out. Most of the cells were triangular in shape with highly refractile granules inside the cytoplasm. A few assumed a more dendritic shape and were found to be in contact with melanocytes. Within 1 week, small clusters of keratinocytes could be observed.

Melanocytes, at 2-3 days, often assumed a fusiform of quadrilateral shape. As time progressed, some melanocytes became more dendritic, while others remained without dendrites, figure 4. A frequency histogram of the distribution of dendrites is shown in fiure 5. Melanin granules were uniformly distributed throughout the cytoplasm, within the cell body and the dendrites.

Some of the melanocytes established contact with keratinocytes through their dendrites. One melanocyte could be in contact with one or more keratinocytes, and one keratinocyte might be in contact with more than one melanocytes. Pigment granules were seen in the cytoplasm of keratinocytes in contact with melanocytes. Two or more melanocytes could be seen in close contact or overlapping each other, figure 20. This most likely represents cell division, where daughter cells remain in contact with each other due to the low motility rate of melanocytes.

The number of attached melanocytes were highly variable and differed between experiments. Even from the same cell suspension, the yields of melanocytes were different. Since there is one melanocyte per 35-40 keratinocytes (Prunieras, 1976), the number of attached melanocytes ranges from one out of 40 to one out of 5 melanocytes planted, similar to those reported by Prunieras (1976) and Cohen & Szabo (1967). The ratio of attached melanocytes to attached keratinocytes were also found to vary from chamber to chamber, and even from different areas within the same chamber.

Changes in Morphology

Control:

As the culture aged, many melanocytes enlarged and became dendritic. However, a large proportion of melanocytes remained in fusiform or rectangular in shape, with short processes. The

majority of cells had 1 or 2 dendrites with very few having secondary branchings. It was observed that melanocytes, in close contact with keratinocytes, were more dendritic, and that they appeared to survive longer than isolated melanocytes. For dendritic melanocytes, age appeared to influence dendritic length. As they were getting older, their dendrites appeared to become longer. They also appeared lighter in color, and more refractile, compared to the younger melanocytes, perhaps a reflection of decrease concentration of melanin granules and a decrease in pigmenting capacity (Klaus,1977). Melanocytes from different guinea pig seemed to have different proportion of dendritic cells.

Since the number of dendrites appears to be influenced by age of the culture, contact with keratinocytes, and the individual animals, comparison of results from separate experiments presented a major problem in analysis. A comparison within an experiment was made. However, for ease of comparison and statistical analysis, the data to be given were taken from pooled results of a single day, Day 5, i.e. 5 days from which the agents were added to the cultures.

Table 2 shows the distribution of melanocytes according to the number of dendrites. Since Day 0 represents melanocytes whose ages range from 4-7 cultured days, the data shown in table 2 (Day 5) are taken from heterogeneous groups of melanocytes whose ages range from 9-12 cultured days, and are taken from different animals. Compared tomelanocytes at Day 0, figure 5,

there were more cells with 1 or 2 dendrites, 36% and 43.7% compared to 19.7% and 9.4% respectively on Day 0. The number of dendrites per 100 melanocytes increased significantly from 48 to 141 (Student T test: P less than 0.001). This tends to confirm the above observation that the number of dendrites increases with age. However, there were only slight increase in melanocytes with 3 or more dendrites. Figure 6 shows tracing of cells from camera lucida. It can be seen that the size and length of dendrites are variable, but most of the melanocytes have 1 or 2 processes.

NGF Group

10 ng/ml:

The appearance of melanocytes incubated in 10 ng/ml NGF was similar to that of the control. Melanocytes were seen in contact with keratinocytes. The amount of melanin granules within the cytoplasm of melanocytes, as judged by the intensity of color imparted on melanocytes, was not differed from that of the control.

Figure 7, 22, 23, represent a serial tracings and photographs of melanocytes, incubated in media containing 10 ng/ml NGF over several days. It can be seen that there is no stimulation of dendritogenesis. In general, the cells showed variation in size and shape, but there was no marked change from control or prior to treatment. The majority of the cells had 1 or 2 dendrites,

with very few having multiple branchings. The dendrites tended to be short, but long processes could, sometimes, be seen.

Table 2 and figure 17 show the distribution of melanocytes into different subgroups on Day 5. A high percentage of the cells are found to have 1 or 2 dendrites, 39.8% and 34.4%, not markedly differed from the control. However, there appears to be more cells that remains without dendrite, compared to control.

The number of dendrites/100 cells of the treated melanocytes was not different from the control, when comparisons were made between melanocytes taken from the same guinea pig and having the same culture age. The values for the control vs the 10 ng/ml NGF were: 145 vs 130, 97 vs 96. However, when comparison was made from the pooled data, there was a significant different between the 2 groups, 141 vs 120 (P less than 0.001). The control group had a larger number of dendrites/100 cells.

50 ng/ml NGF:

This represents the largest group of melanocytes treated. There appeared to be no difference between the treated and the control groups, with the majority of cells having 1 or 2 dendrites. Melanocytes were seen to interact with keratinocytes in a normal fashion. Pigmenting ability and melanin content seemed unaffected by incubating in 50 ng/ml NGF.

Figure 8 shows tracings from treated melanocytes over a period of time. The morphology appeared not to differ from the

control, figure 6. Figure 9, 10, 11, 24, 25, 26, 27 are a series of photographs and tracings taken at various intervals after the addition of NGF.

For a small number of cells, there appeared to be minor change in shape, primarily ruffling of the cell membrane. These changes were transient and the cell membrane resumes its smooth contour within 4-6 hours. These changes were not, however, observed in all melanocytes, probably reflecting the heterogeniety of the melanocyte population. Overall, there was no significant alteration in the shape of melanocytes or in lengthening of the dendrites.

Figure 17 and table 2 represent the pooled data on Day 5. The majority of the cells were rounded, 29.6%, or having 1 or 2 dendrites, 37.4% and 27.7% respectively. Similar to the 10 ng/ml group, there were increasing proportion of cells with no dendrites, compared to control. As in the control, multiple branchings of dendrites were rarely observed.

When melanocytes were matched for age and cell sources, there was no different between the control and the 50 ng/ml group in the average number of dendrites/100 cells. The values for the control vs the 50 ng/ml NGF were: 97 vs 89, 106 vs 109, 124 vs 130, 145 vs 126. This supported the above observation that NGF, at 50 ng/ml, did not significantly affect the dendritogenesis. However, when the pooled data were compared, NGF seemed to depress dendritogenesis. The average number of dendrites per 100 cells of the control vs the 50 ng/ml NGF were: 141 vs

111, with a P value less than 0.001.

100ng/ml NGF:

The cell cultures maintained at this concentration were not differed from the control or the other 2 NGF treated groups. No apparent toxic effect was observed at this concentration. Melanocytes continued to interact with keratinocytes and a few were observed to be capable of cell division.

The majority of cells had 1 or 2 dendrites of varying length. A large proportion had small processes, whose length were not long enough to qualify as dendrite, as defined in this experiment. Very few dendritic cells were observed and multiple branchings were rarely seen. Figure 12, 28, 29 show a serial tracings and photographs of melanocytes incubating in 100 ng/ml NGF. They were comparable to the control, figure 6, except for having more cells with shorter processes.

The distribution of melanocytes into subgroups can be seen in figure 17 and table 2. The small number of melanocytes, 146, does not represent the total number of cells in this group, but reflects the number of melanocytes counted on this particular day only. Most of the cells were rounded or had 1 or 2 dendrites. Only 2.1% of the cells had 3 dendrites and none possessed more than 3 dendrites.

The average number of dendrites/100 cells between the control and this group were comparable, provided that they were

taken from the same animal, and were of the same culture age. The values for the control vs the 100 ng/ml NGF were: 73 vs 70, 97 vs 93. This appears to correlate well with daily observations and tracings obtained. As in the previous groups, when comparison was made from the pooled data, NGF appeared to depress dendritogenesis. The average number of dendrites/100 cells of the control and the 100 ng/ml NGF were 141 vs 98 respectively. The difference was significant to P less than 0.001.

DBcAMP-Theophylline:

There were definite changes when melanocytes were exposed to the DBcAMP-Theophylline mixture. While the number of melanocytes progressively decreased with time in all cultures, those maintained in DBcAMP-Theophylline decreased at a less rapid rate. They appeared to survive longer and looked "healthier". There was no observable difference in the number of dendrites innervating keratinocytes. Nevertheless, there seemed to be an increase in the amount of melanin granules, as determined by the color of melanocytes.

Within 2-3 days of incubating in DBcAMP-Theophylline, the cells became more dendritic, although not all of the cells responded. Those with only 1 or 2 dendrites sent out multiple branchings and the dendrites were lengthened to 1-3 times the cell body. Traces of fragmented dendrites were sometimes seen attached to the coverslips, without detrimental effect to the

parents.

Figure 13, 14, 15, 30, 31 depicted melanocytes as they were seen under phase contrast microscopy. Figure 17 and table 2 show the frequency distribution of the treated melanocytes. While the majority of the cells still had 1 or 2 processes, these processes were longer in length and more complex. There was a significant shift of cells into groups with 3 or more dendrites, 14.6% and 6% respectively.

When the number of dendrites/100 cells was compared to the control, there was a significant increase in the treated group (P less than 0.001). Using the pooled data on Day 5, the average number of dendrites/100 cells in the DBcAMP-Theophylline group was 169, compared to 141 for the control. The increase was more pronounced when comparison was made within each experimental group, control vs DBcAMP-Theophylline: 97 vs 193, 130 vs 296 (after 10 days).

The stimulation of dendritogenesis by DBcAMP-Theophylline was reversible. When the cultures were washed twice with normal medium, and subsequently incubated in Eagle MEM with 30% fetal calf serum, melanocytes were found to lose the complexity of the dendrites. The dendrites became shorter and were reduced in numbers. See figure 15 H and figure 32.

Table 2 and figure 17 summarize all the results from different groups on Day 5. Figure 18 is a graphic representation of the average number of dendrites/100 cells of the various

groups on Day 5. It should be borne in mind that these were taken from the pooled data, from a very heterogeneous groups of melanocytes, and may not have any significance in reality. Nevertheless, from this data, DBcAMP-Theophylline stimulated dendritogenesis, whilst NGF depressed it. As the concentration of NGF increased, the average number of dendrites per 100 cells decreased.

Cell Size:

Age seemed to have an effect on cell size in the first week. They enlarged as they grew older. However, they did not continue to enlarge after the first week. Cell size was measured as previously described in the Materials and Methods.

Table 3 summarizes the cell size from all the different groups. Both absolute and relative values were given. However, for ease of comparison, each absolute value was converted into relative value, taking the control average as 100.

The relative values of the 10, 50, and 100 ng/ml NGF were not significantly different from that of the control, 107.3 (P less than 0.40), 108.6 (P less than 0.30), and 104 (P less than 0.60) respectively. However, those cells incubating in DBcAMP-Theophylline appeared to have grown in size, to the extent that some had to be photographed at 160 X rather than the usual 400 X. They were found to be 1.6 times larger than the control (P less than 0.001). When they were compared to matched control, the ratio increased to 1.7-1.8.

DISCUSSION

GENERAL:

The appearances of the cultures are similar to those reported by Cruickshank et al (1960), Klaus (1969), Cohen & Szabo (1968), Prunieras (1976). The number of melanocytes recovered are also similar to the ratio reported by Cohen & Szabo (1968), and Prunieras (1976). Thus, the overall qualities of the cultures are comparable to the earlier reports. However, there is one minor difference that merits discussion.

The number of dendrites between this experiment and that reported by Cruickshank et al (1960) are different. He reported that there were numerous dendritic melanocytes with extremely long processes, whereas in this experiment, most of the cells had 1 or 2 short and simplified dendrites. Since he did not report the percentage of dendritic melanocytes, it could not be certain whether the majority of his cells were dendritic. Furthermore, the melanocyte population that he mentioned appeared rather suddenly. Secondly, the age of the culture was not specified. The dendritic cells may represent older cells compared to this experiment. Fritsch (lecture at Yale) had used the terms "epitheloid" and "dendritic" melanocytes to denote 2 types of cells and suggested that they might have different mitotic processes. It was also speculated that the dendritic cells were more metabolically active and more sedentary (Drzeiwieki et al,1977). Thus, the difference in the number of

dendrites could be due to the different proportion of dendritic cells found in different guinea pig strains, or to the heterogeneity of the recovered melanocytes. Since each experimental group also serves as its own control, the interpretation of the results is valid and should be applicable to other melanocytes, regardless of the number of dendrites originally..

DENDRITOGENESIS:

In his doctoral thesis, Chen (1974) demonstrated that DBcAMP, an analogue of cAMP, caused an increase in cell size and in the number of dendrites of goldfish melanocytes. Kitano (1973; 1976a) reported a similar response in human melanocytes in vitro. By incubating human melanocytes in 1 mM DBcAMP, he noted an increase in number, length, and complexity of the dendrites and cell size. However, DBcAMP may not be suitable to pretreat the cells before elutriation because it also appears to stimulate keratinocyte proliferation (Greene,1978).

Other agents that proved to be stimulatory include melanocyte stimulating hormone (MSH), theophylline, and UV irradiation. Although MSH alone does not cause a significant change in human melanocytes, together with theophylline, they cause an enlargement and also stimulate dendritogenesis in melanocytes (Kitano,1976b). However, Hirobe (1978) was able to show that MSH stimulated dendritogenesis in melanocytes of newborn mice. In addition to increasing the number of melanocytes, UV irradiation causes the cell to become enlarged and more

dendritic (Rosdahl & Szabo,1978).

In 1975, Nikodijevic et al reported a transient several fold increase of cAMP in the freshly excised rat superior cervical ganglia when NGF was added to the culture medium. This transient increase in cAMP was recently confirmed by Skaper et al (1979) when they reported a transient 5 fold rise in cAMP in the NGF treated embryonic chick dorsal root ganglia. Since we know that cAMP analogue, DBcAMP, stimulates dendritogenesis in melanocytes, NGF, by virtue of its effect on cAMP level, might also stimulate dendritogenesis in melanocytes in vitro.

From the data obtained, NGF does not stimulate dendritogenesis in guinea pig melanocytes. In serial tracings and daily observation, no major change in the number, length, and complexity of the dendrites was detectable. Any delayed effect was not detected when the cells were incubated in NGF for up to 10 days. When these cells were subsequently incubated in DBcAMP-Theophyline, they became dendritic, figure 19. This rules out the argument that the cultured melanocytes may not be able to form dendrites.

Transient ruffling of the cell membrane was noted to occur within 2 hours and disppeared in 4-6 hours, when cells were incubated in 50 ng/ml NGF. Because this effect is seen in only a few cells, it is not certain whether this represents real or just a coincidental finding. Nor is it clear whether this is due to the "shock" from media change, or to the NGF. Media

change in the control does not lead to ruffling of the cell membrane. The addition of NGF may alter the pH or other physical properties of the culture media, causing the membrane to ruffle. Alternatively, the addition of NGF leads to a transient increase in cAMP, as seen in embryonic chick dorsal root ganglia (Skaper et al,1979). The magnitude and duration of increased cAMP level may not be large enough to stimulate dendritogenesis, comparable to that seen in DBcAMP-Theophylline, and hence, results in only ruffling of the membrane.

When each subset of the experiment is compared to itself, for average number of dendrites/100 cells, there is no significant difference amongst the control and the NGF treated group. However, when the pooled data from a particular day, Day 5 - the fifth day after the addition of NGF, the results are surprising. While the effect of DBcAMP-Theophylline is confirmed in guinea pig melanocytes, NGF, at all concentrations, seems to depress dendritogenesis. The average number of dendrites per 100 cells appears to be inversely related to the concentration of NGF. The number of the 10 ng/ml NGF is 120, while that of the 100 ng/ml NGF is only 98, compared to 141 for the control. Whether this represents a true depressant effect of NGF is unclear, since melanocytes from different subsets are not of the same culture age and are taken from different guinea pigs. Furthermore, the pooled data are taken from a single day, and that not all of the cultures are examined on the fifth day of treatment. Nevertheless, the data are presented, with the

aforementioned criticisms in mind, because it is conceivable that the differences in each subset may not be significant due to the small number of cells in each group. When these differences are pooled together, they become significant.

CELL SIZE:

Agents that can increase melanocyte size have been reported. In general, it appears that agents that stimulate dendritogenesis in melanocytes also cause the cells to become enlarged. Kitano (1976a; 1976b) demonstrated the enlargement of melanocytes *in vitro* when they were incubated in DBcAMP or in MSH and theophylline. These cell enlargement effects were found to be reversible when the cells were subsequently incubated in normal media. In addition to stimulating dendritogenesis, UV irradiation results in increasing the cell size of mouse melanocytes.

In general, the average cell size in this experiment is larger than that previously reported by Riley (1975), 4400 vs 2100 um^2 respectively. The observation and the measurements show that NGF does not cause the cells to become enlarged, while cells incubated in DBcAMP-Theophylline become larger. Since agents that stimulate dendritogenesis also cause enlargement and since NGF does not stimulate dendritogenesis, it is not surprising that NGF has no cell enlargement effect.

The results obtained are contrary to the hypothesis that NGF would stimulate dendritogenesis and increase the cell size

in melanocyte, which are also neural crest derivatives. There could be several reasons for these results.

First, NGF may not have any effect at all on normal melanocytes. Although receptors for NGF have been found on human melanoma cells, such receptors have not yet been reported in normal melanocytes. The finding of the NGF receptors in human melanoma may be a unique feature of the transformed cells, as in the mouse sarcoma 180, and is not to be seen in a normal melanocyte. Secondly, the amount of NGF used may not be enough. The maximal concentration used, 100 ng/ml, may not reach the minimal level necessary for stimulatory effect.

In addition, the melanocytes in this experiment are differentiated cells from adult animals. Levi-Montalcini (1966) reported that NGF could stimulate sensory ganglia of newborn mice but had no effect in adult animals. Thus, it is conceivable that melanocytes from adult animals lose their responsiveness to NGF. Another alternative is that NGF is inactivated in this experiment. There may be certain protease(s) in the serum that can degrade and inactivate the NGF (manufacturer communication). Adsorption of NGF to the glass Cruickshank chambers could also reduce NGF activity.

Does NGF have any effect on normal guinea pig melanocytes? This thesis only focuses on morphological change. Changes in metabolic or enzymatic activities or possibly in cell turnover could have occurred without accompanying morphological change. As melanocytes cannot be grown in serum-free medium (Fritsch:

personnal communication), it would be interesting to see if NGF can serve as a "trophic factor" in place of serum. It is conceivable that melanocytes require a small amount of NGF and this requirement is fulfilled by the small amount of NGF present in the serum, table 1. Future studies in these areas, such as attempts to grow melanocytes in serum-free media, supplemented with NGF, are necessary before we can understand why melanoma cells produce NGF and have NGF receptors, and whether the "autostimulatory theory" put forth by Sherwin et al (1979) is correct.

As previously mentioned, the purpose of this thesis is to establish any morphological effect the NGF may have on normal melanocytes and whether the information gained can be used to improve the method of culturing melanocytes. It can be concluded that NGF, at the concentrations used, does not stimulate dendritogenesis, nor does it have any effect on cell size. There is a possibility that NGF may depress dendritogenesis, but more studies are required before this statement can be held true, as such effect is not seen in each subset. Since NGF does not have observable stimulatory effect, it is unlikely that it will prove useful in improving the culturing technique.

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Table 1

NGF ACTIVITY IN SERA#

ANIMAL	NGF ACTIVITY (biological units ml^{-1}
Mouse, male	10-20
Mouse, female	0.7
Human	1.5
Pig	0.2-0.4
Horse	0.1-0.5
Fetal Calf	0.1-0.2
Cockerel	0.2

Taken from Banks et al, 1973

Table 2

Average Number of Dendrites/100 Cells Taken from Day 5

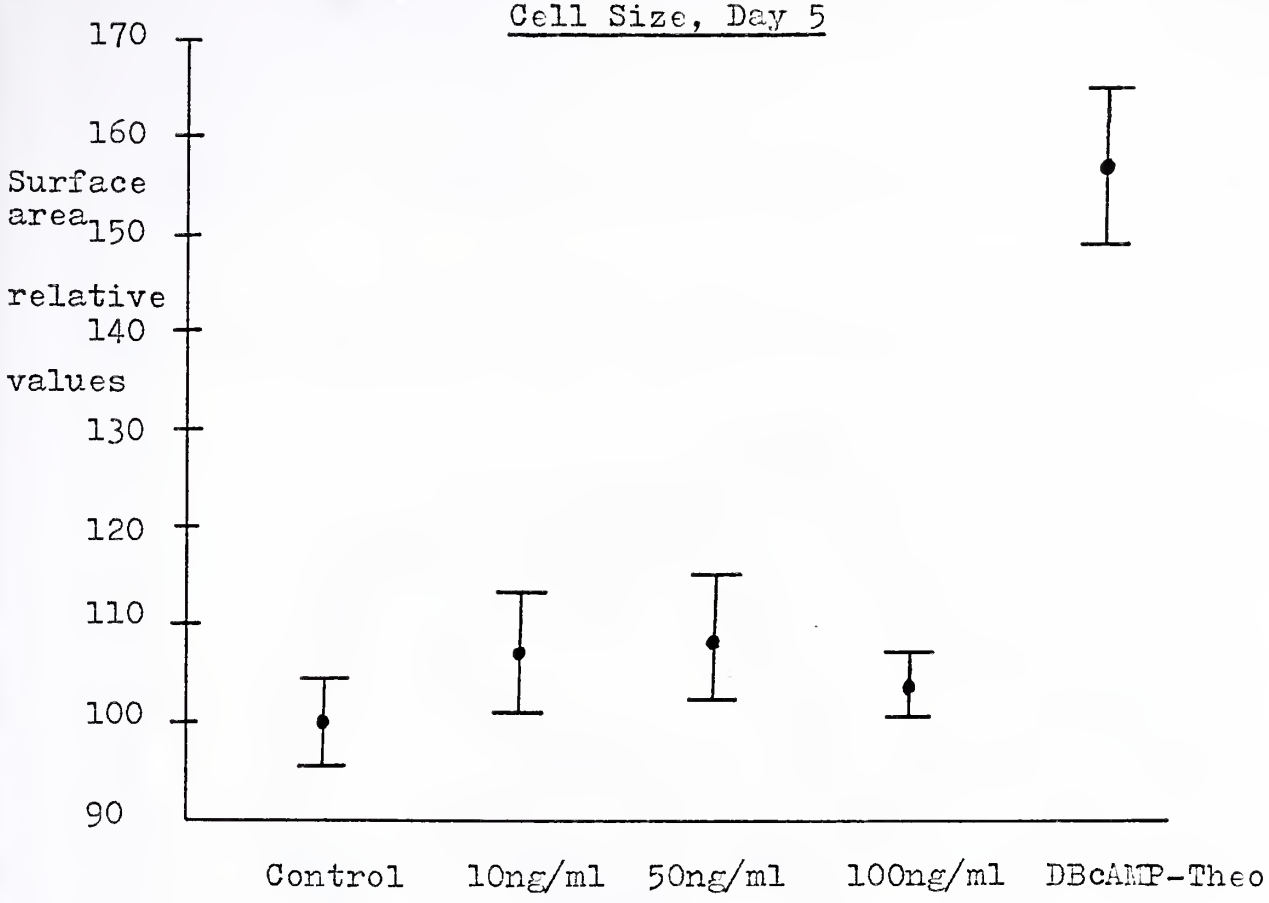
	CONTROL		10 ng/ml		50 ng/ml		100 ng/ml		DBcAMP-Theo	
#Dendrites	#	%	#	%	#	%	#	%	#	%
0	124	14.8	88	21.9	264	29.2	47	32.2	60	14.4
1	302	36	160	39.8	338	37.4	58	39.7	117	28.1
2	367	43.7	138	34.4	250	27.7	38	26	156	37.4
3	35	4.1	13	3.2	42	4.6	3	2.1	59	14.1
more than 3	12	1.4	2	0.5	10	1.1	0	0	25	6
Total	840	100	401	100	904	100	146	100	417	100
Aver.# dend/ 100 cells	141.54		120.44		111.06		97.94		169.3	
S.D.	84.14		83.55		91.79		81.76		107.05	
S.E.	2.9		4.17		3.05		6.76		5.24	

Student T Test

Control	T	P
vs 10 ng/ml	4.25	less than 0.001
vs 50 ng/ml	7.38	less than 0.001
vs 100 ng/ml	5.86	less than 0.001
vs DBcAMP-Theo	5.16	less than 0.001

Table 3

Cell Size, Day 5



Surface Area

	Absolute values um^2	Relative values	T tests
Control	4469	100.00 ± 4.40	
10 ng/ml	4741	107.29 ± 6.40	< 0.40
50ng/ml	4800	108.61 ± 6.31	< 0.30
100 ng/ml	4549	103.98 ± 3.44	< 0.60
DBcAMP-Theo	7015	157.29 ± 8.54	< 0.001

Figure 1. A schematic representation of NGF polypeptide.

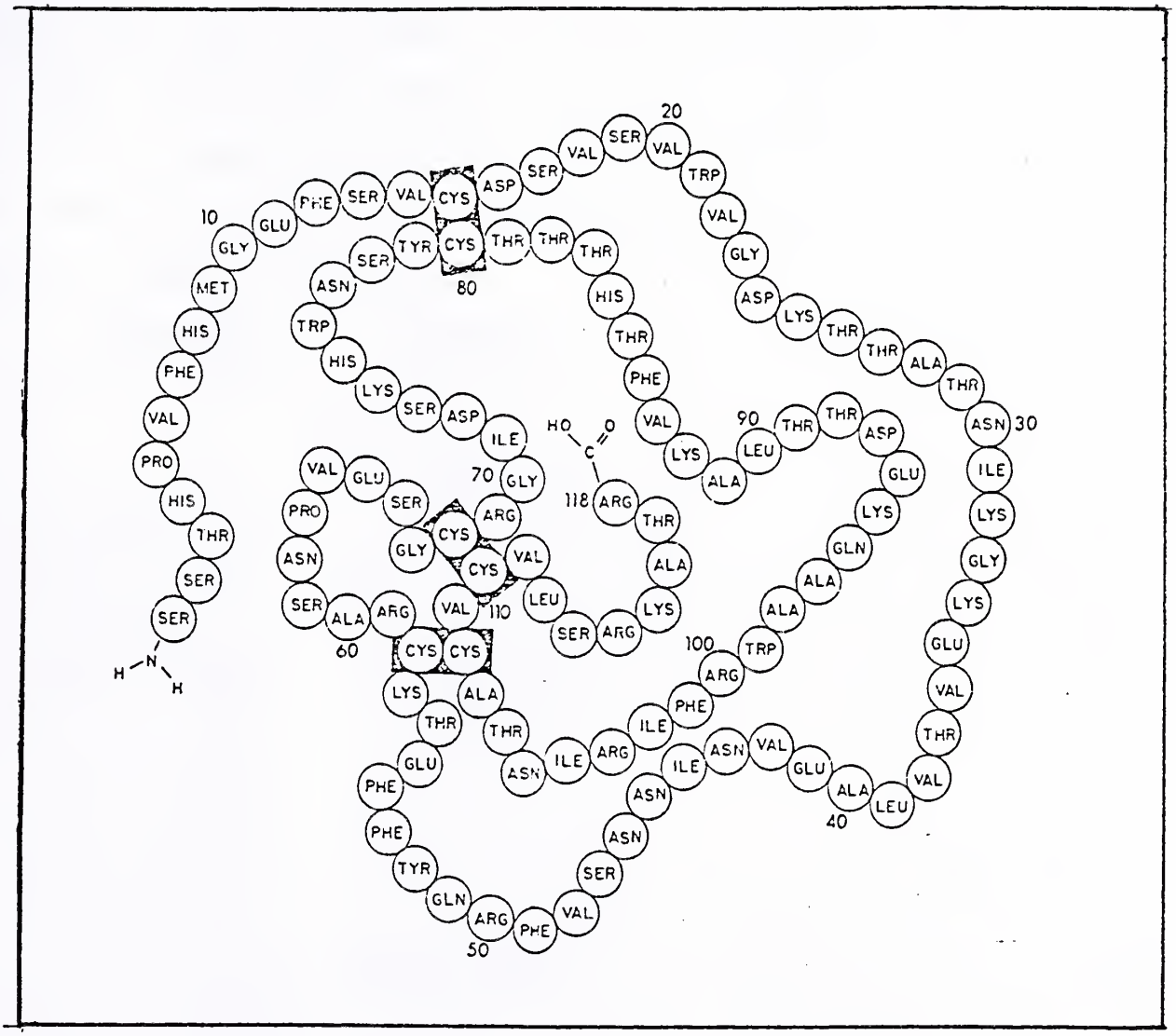


Figure 2

A SCHEMATIC DIAGRAM OF NGF MOLECULE

It is hypothesized that the storage form of NGF, 7S NGF, is a complex of 2.5S NGF, 2 gamma, and 2 alpha subunits. The gamma subunit is an enzyme with arginine esteropeptidase activity. Two gamma subunits attach themselves to a proNGF polypeptide chains and cleave the proNGF to generate the active dimer, a 2.5S NGF. Unlike other enzymes, the gamma subunits remain attached to their end products. Together with 2 alpha subunits, they protect the 2.5S subunit from further degradation by other enzymes (Mobley et al,1978).

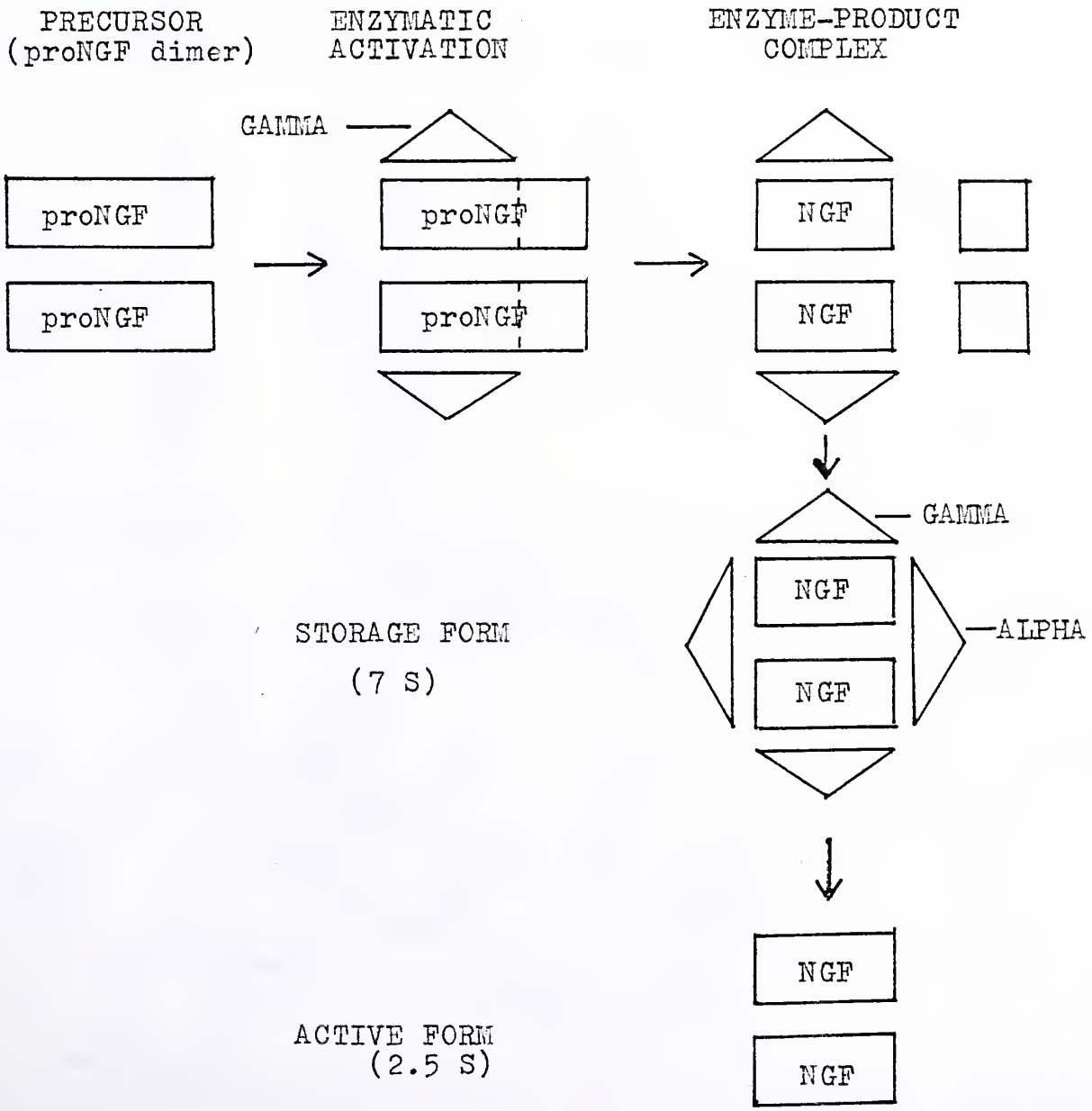
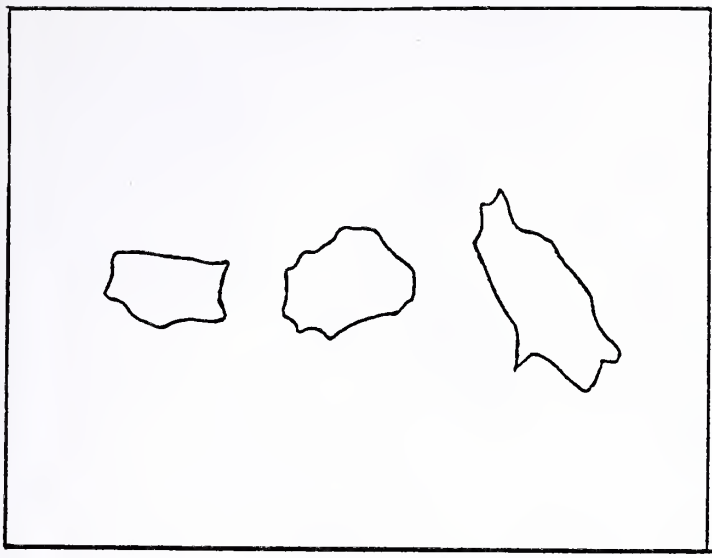
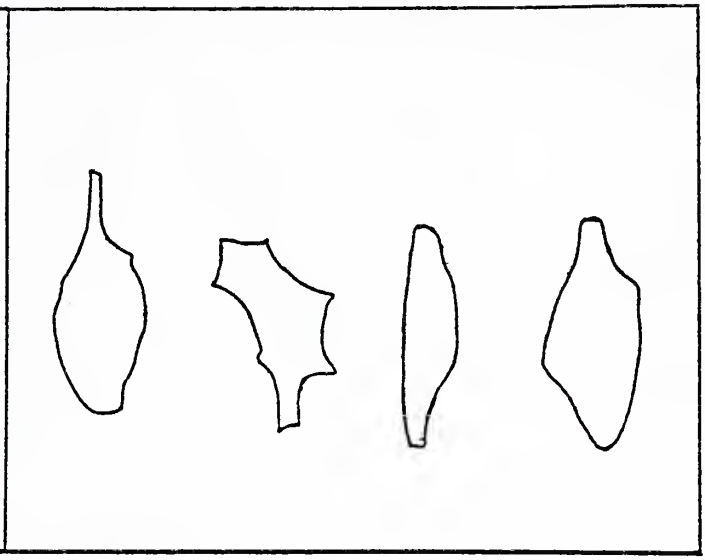


Figure 3. Categories according to the # of DENDRITES

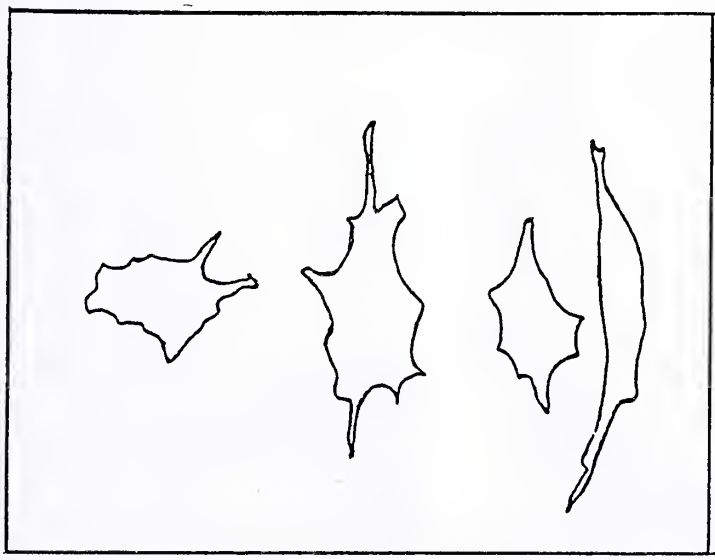
Rounded



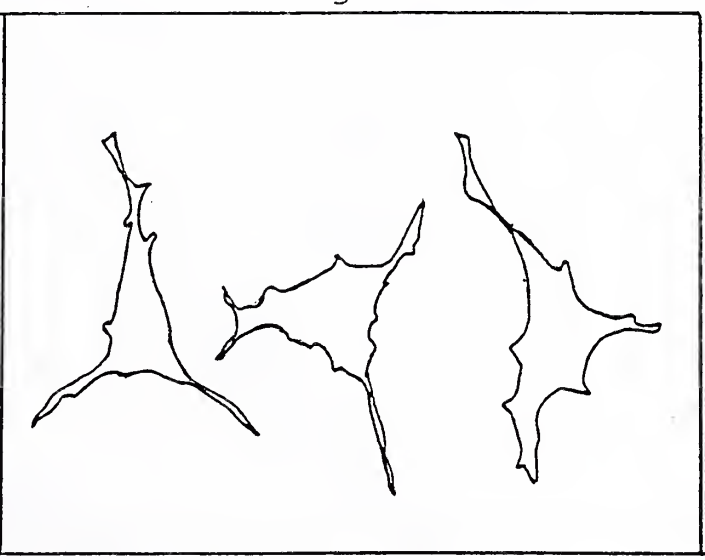
1



2



3



More than 3

Figure 4. Tracing of cells at Day 0 (3-7 culture days)

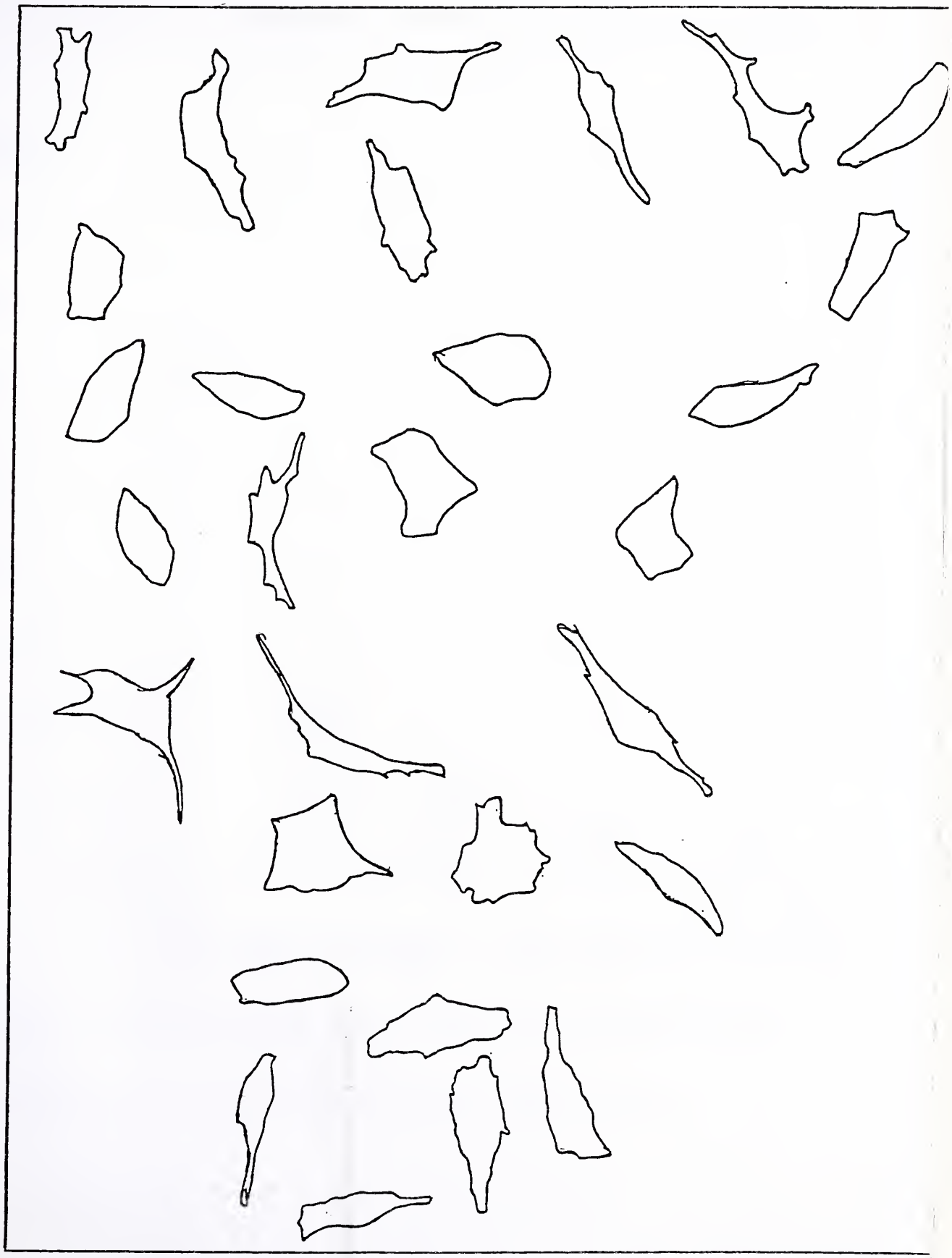
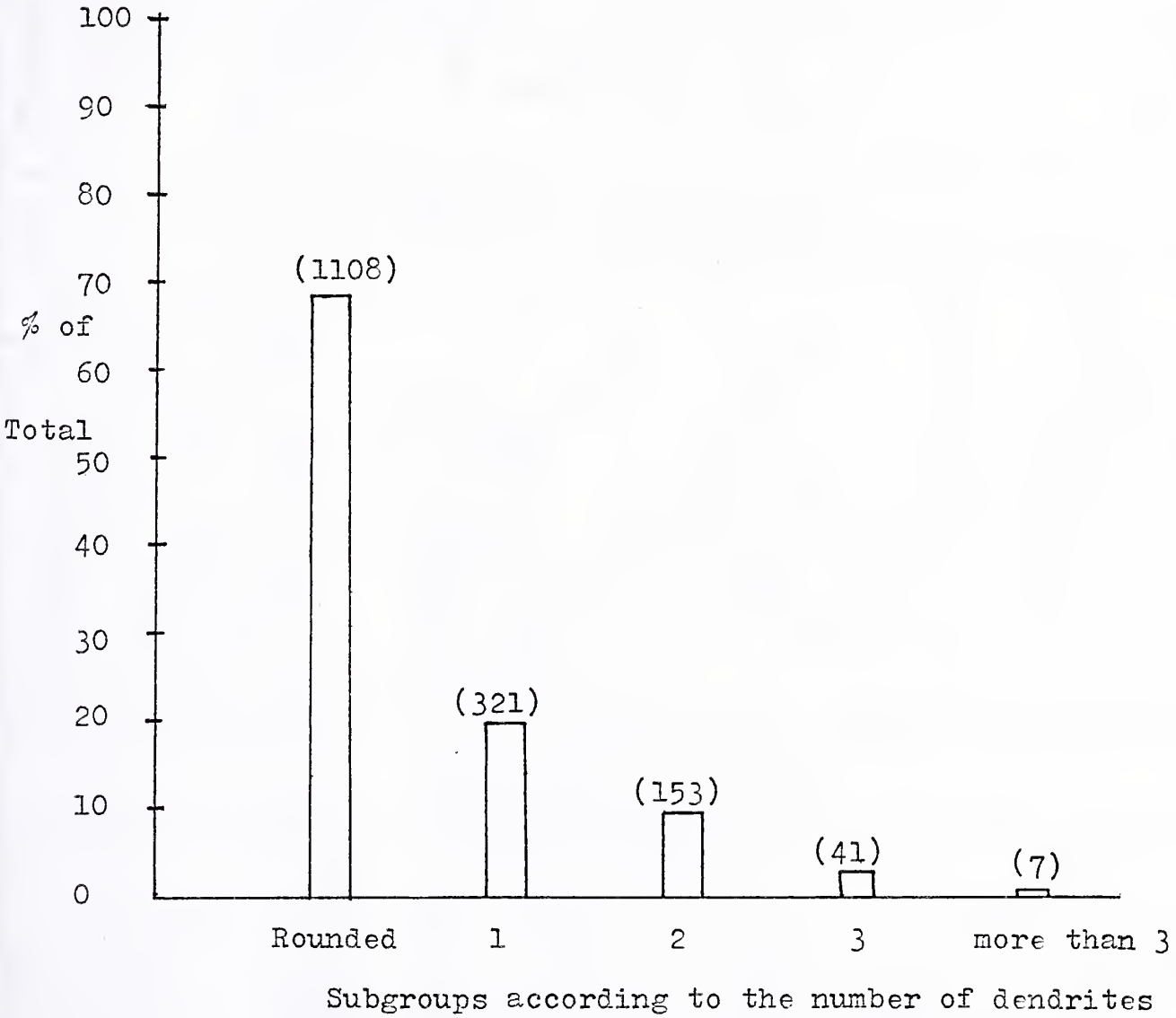


Figure 5

Distribution of Melanocytes according to Dendrites, Day 0

(4-7 culture days)

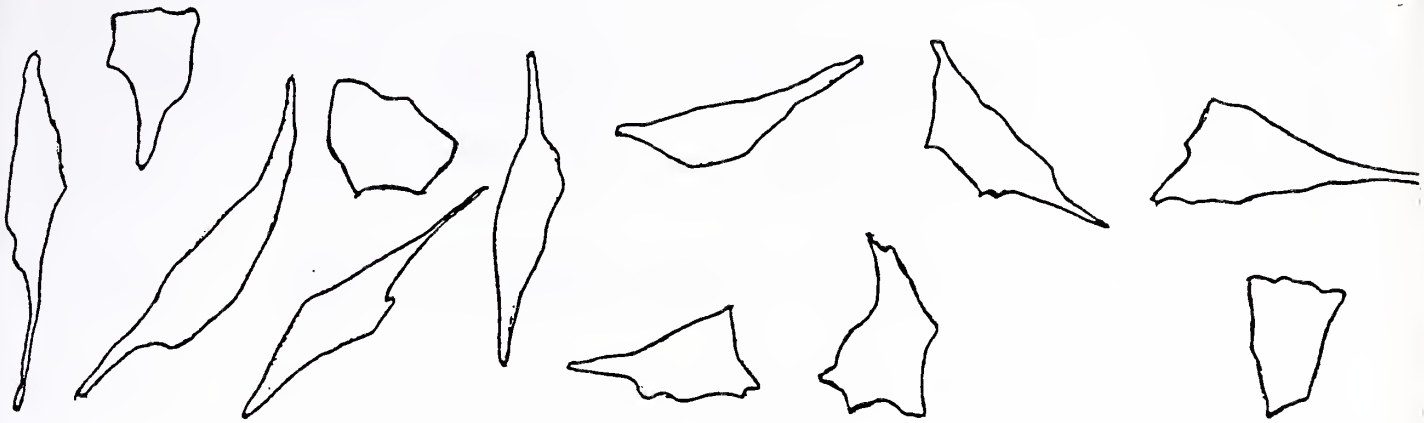


Absolute numbers of each group are given in parenthesis.

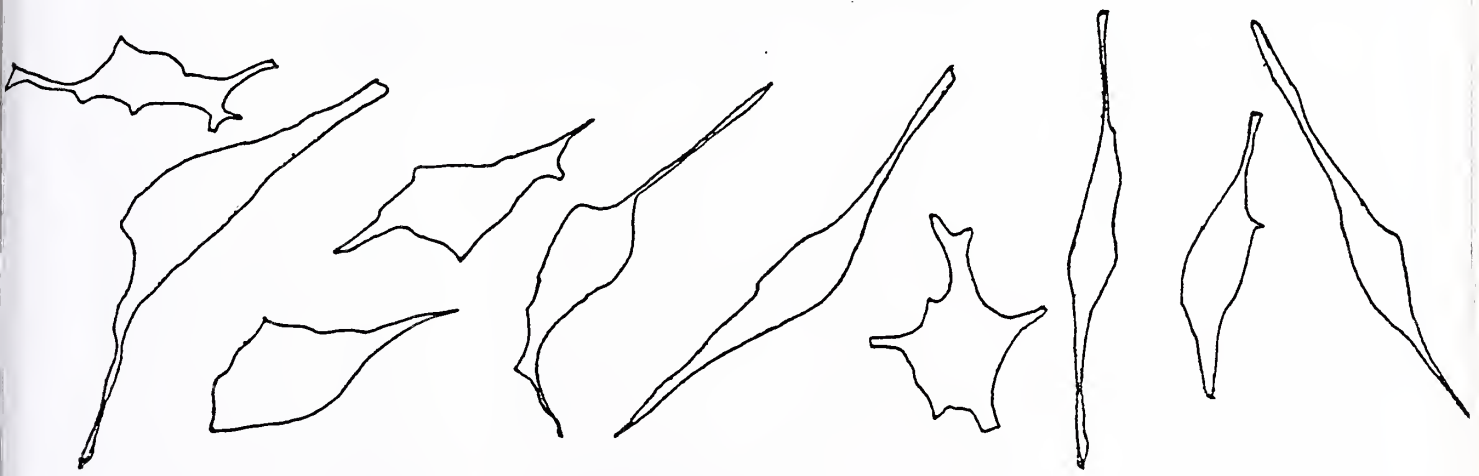
The average number of dendrites/100 cells is 47.6

Figure 6. Serial tracings of control.

Day 1 (5 culture days)



Day 3 (7 culture days)



Day 5 (9 culture days)

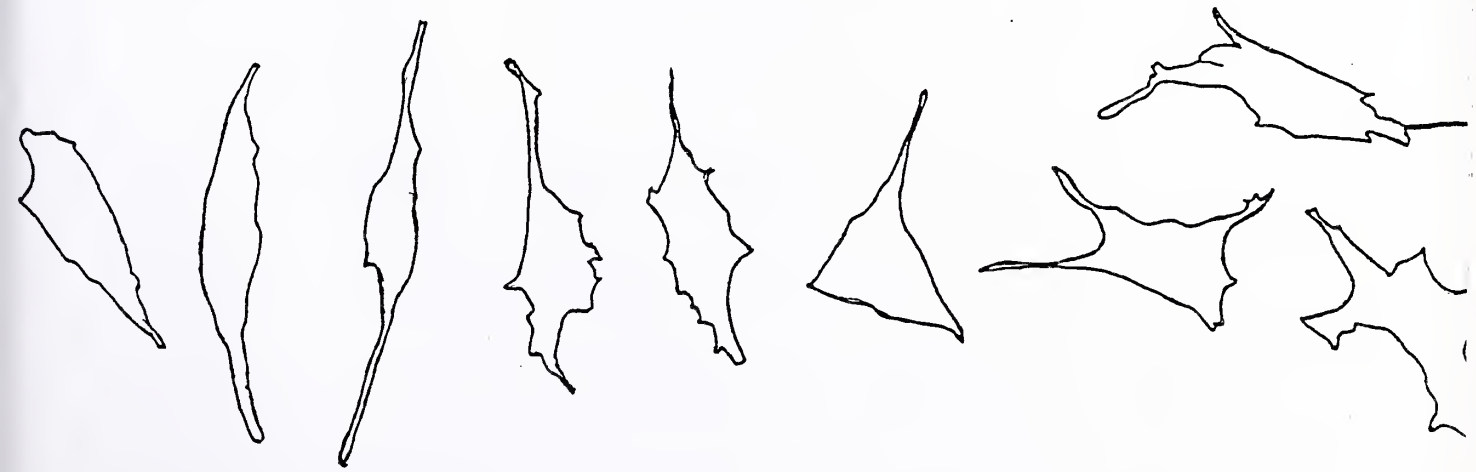


Figure 7. Serial tracings of cells incubating in 10 ng/ml NGF.

A. Day 1, 5 culture days.

B. Day 2, 6 culture days.

C. Day 3, 7 culture days.

D. Day 5, 9 culture days.

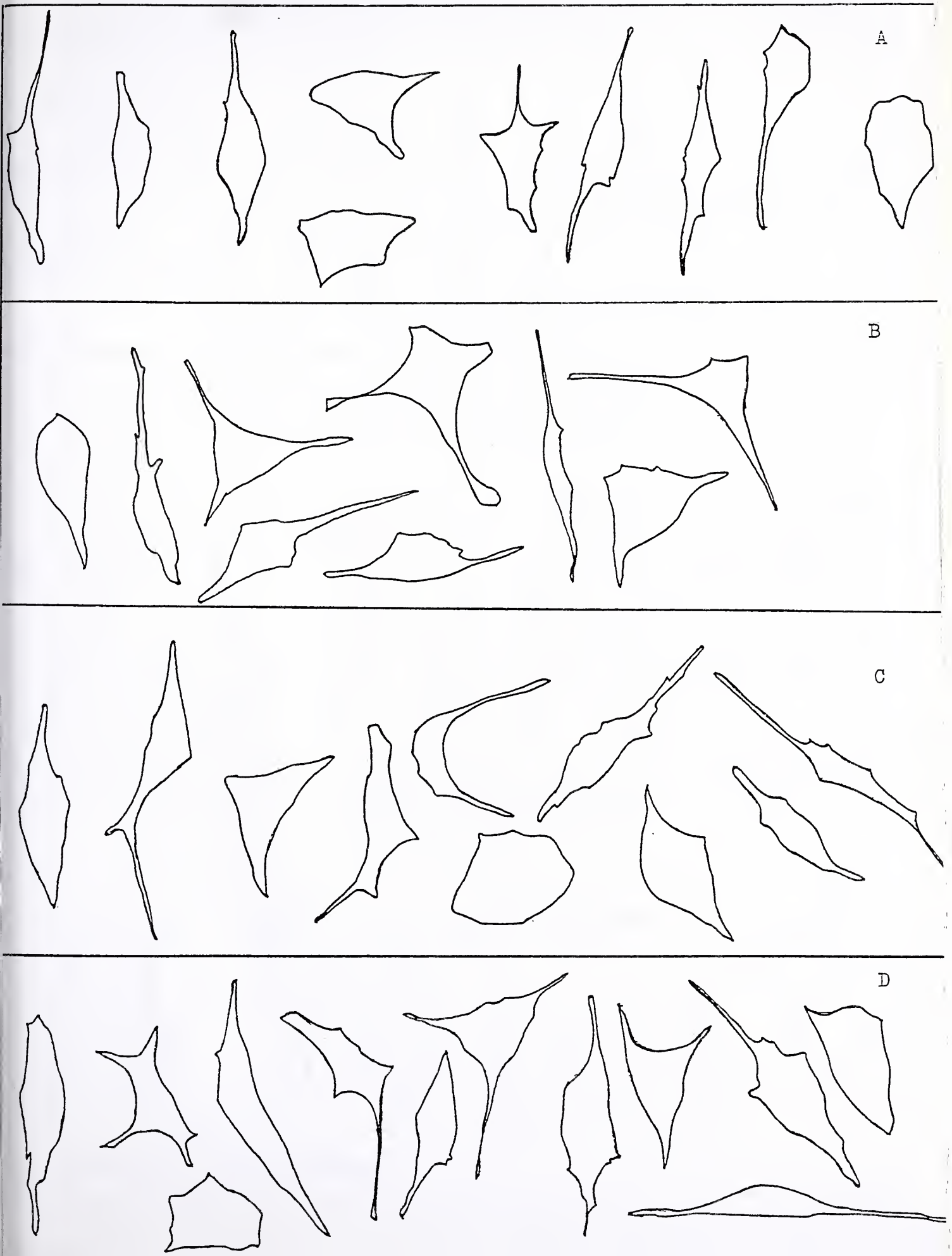
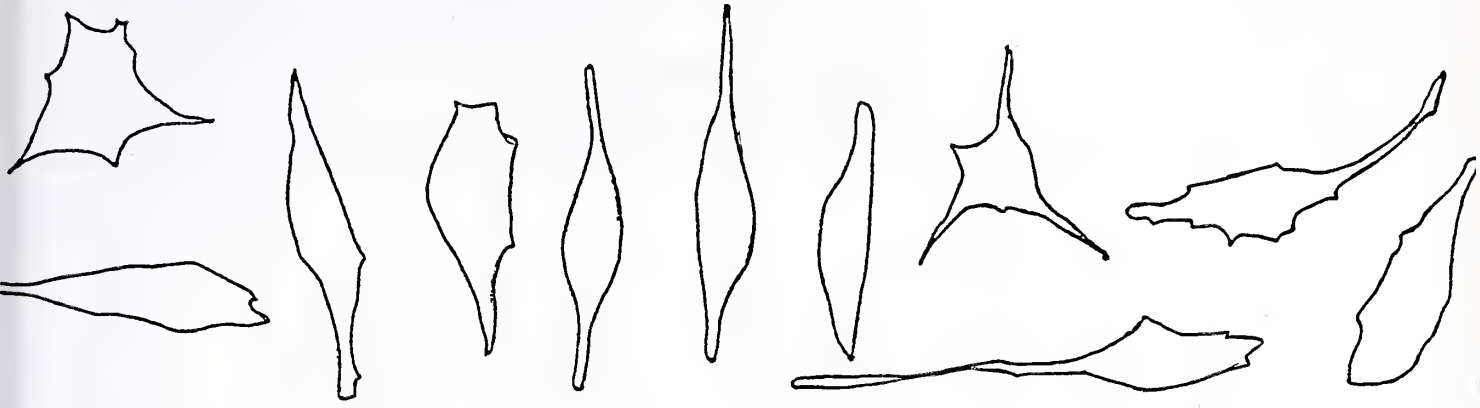
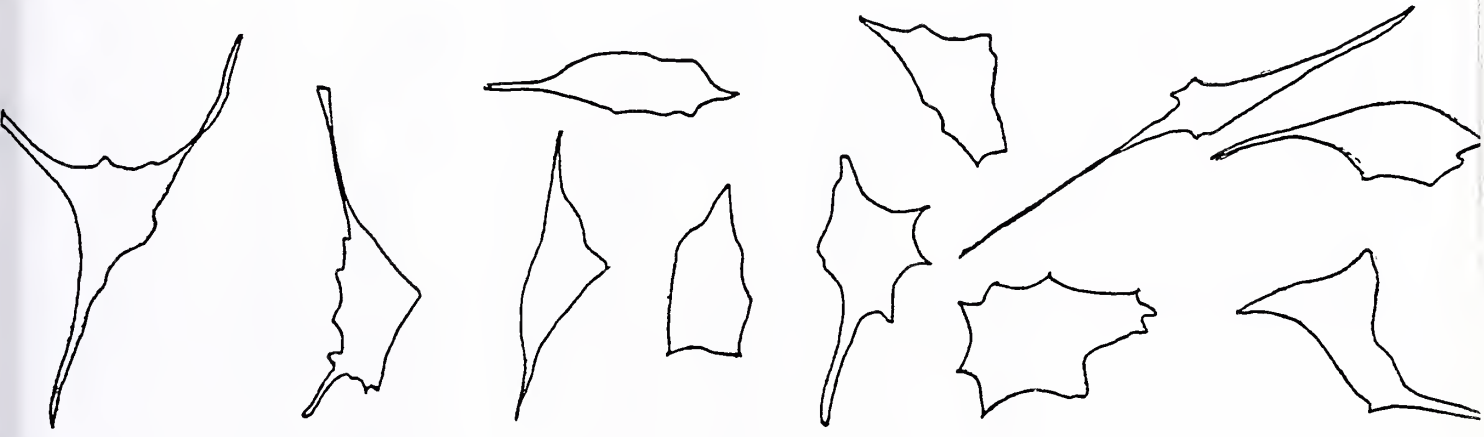

A
B
C
D

Figure 8. Serial tracings of cell incubated in 50 ng/ml NGF

Day 1, 5 culture days



Day 3, 7 culture days



Day 5, 9 culture days

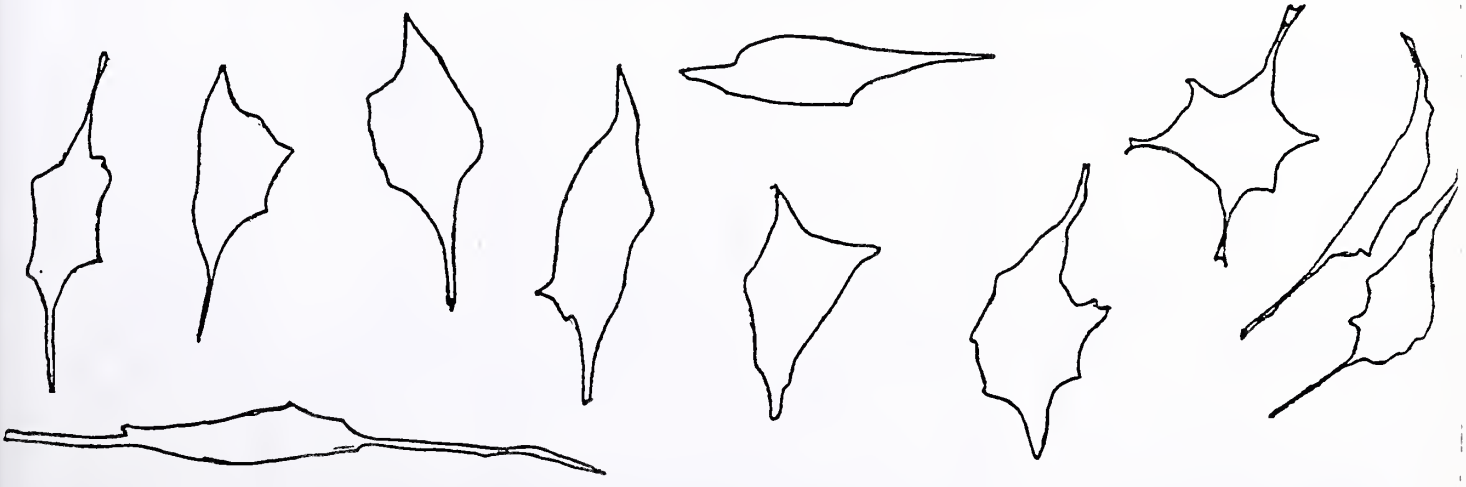


Figure 9. Serial tracings of cells incubating in 50 ng/ml NGF. Note ruffling of the membrane in a and b.

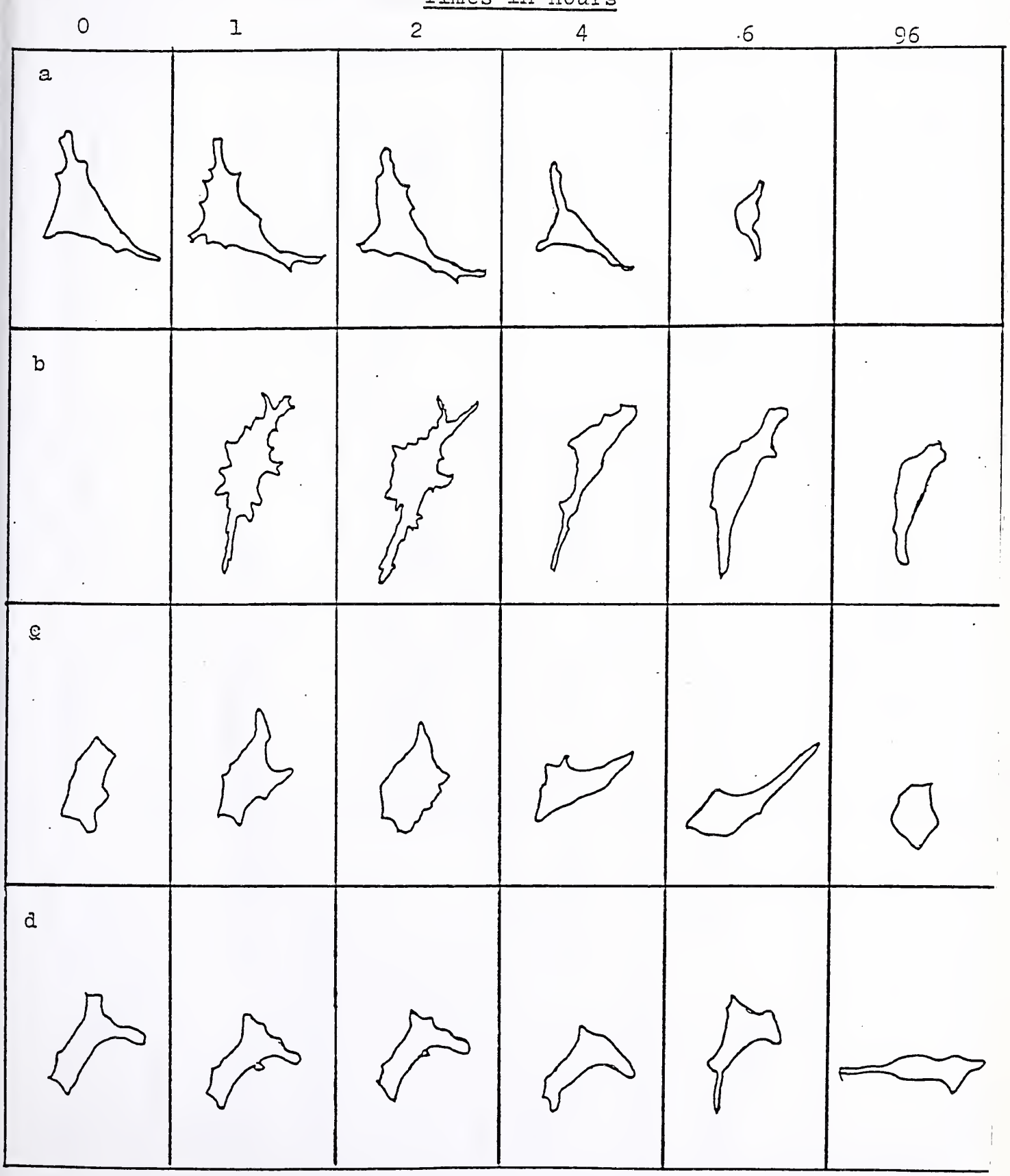


Figure 10. Serial tracings of cells incubating in 50 ng/ml NGF.

Times in hours

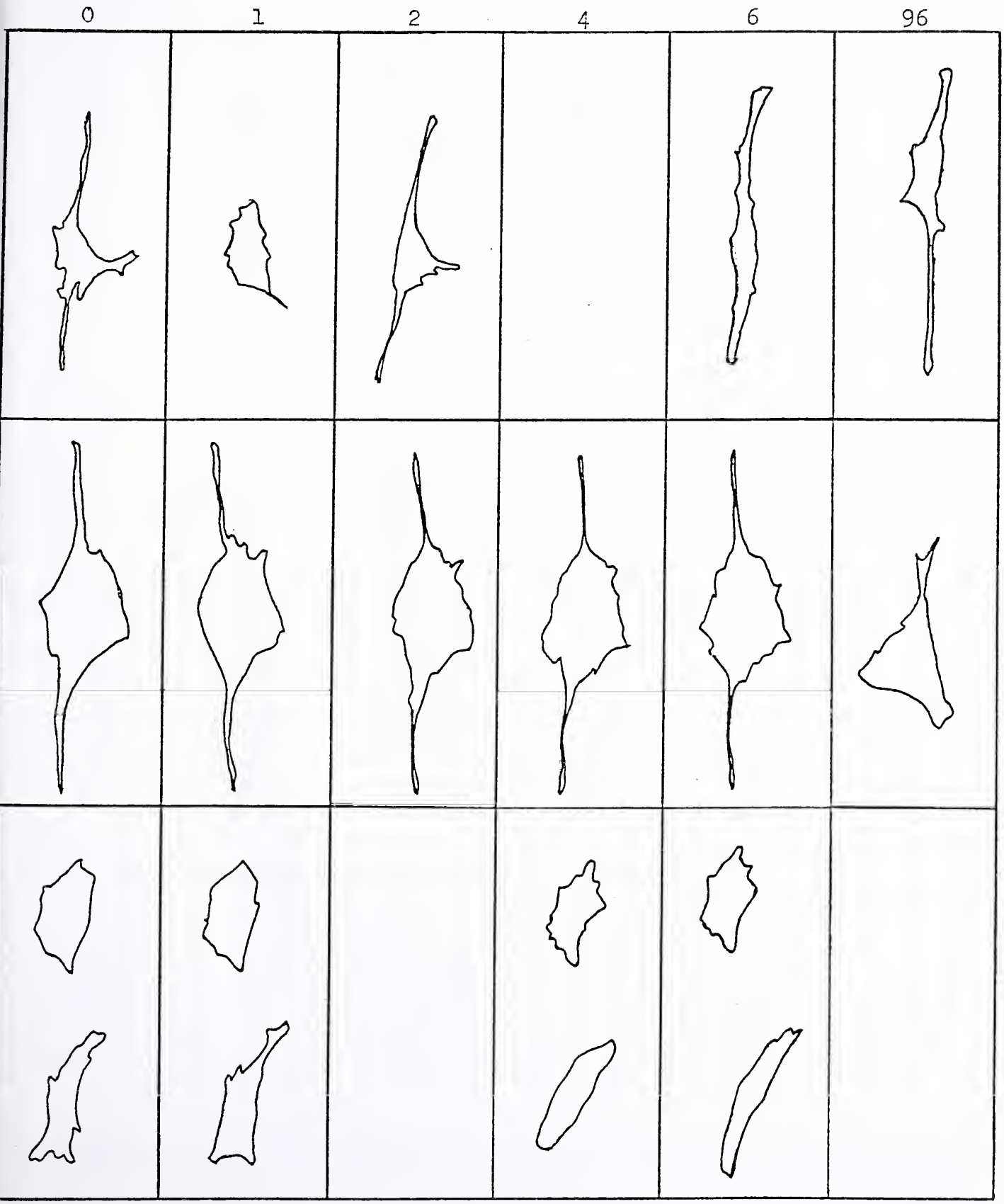


Figure 11. Serial tracings of cells incubating in 50 ng/ml NGF

Times in hours

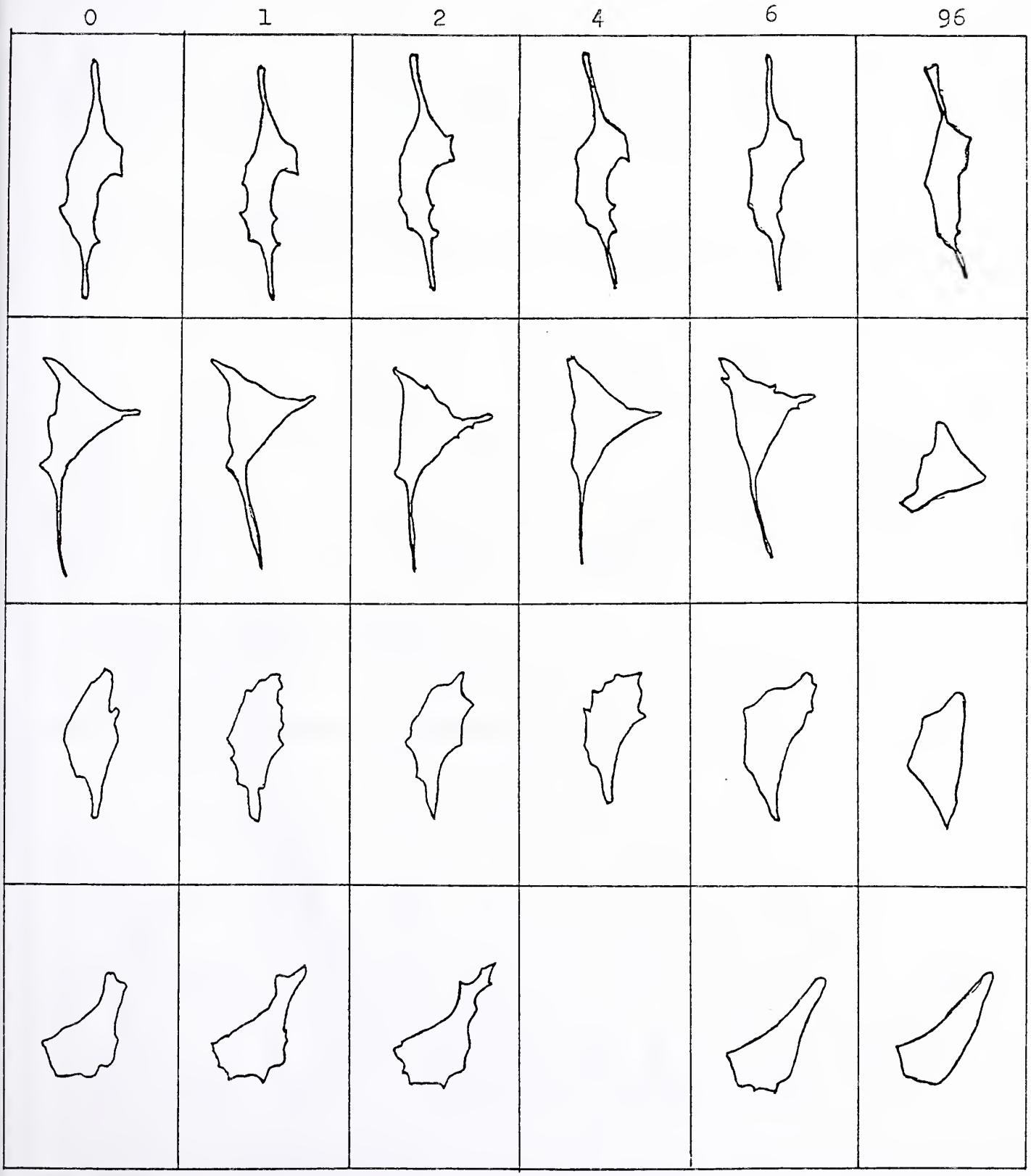
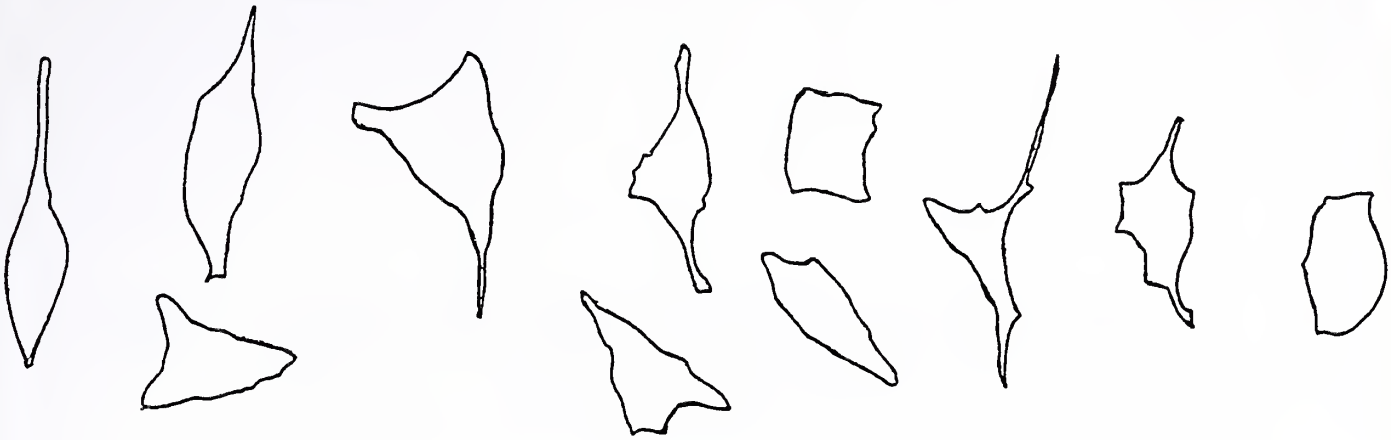
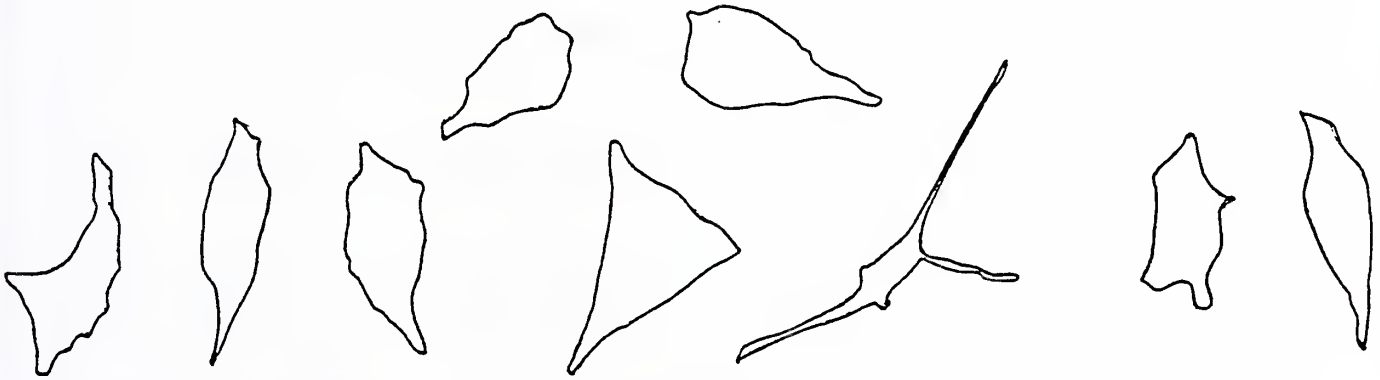


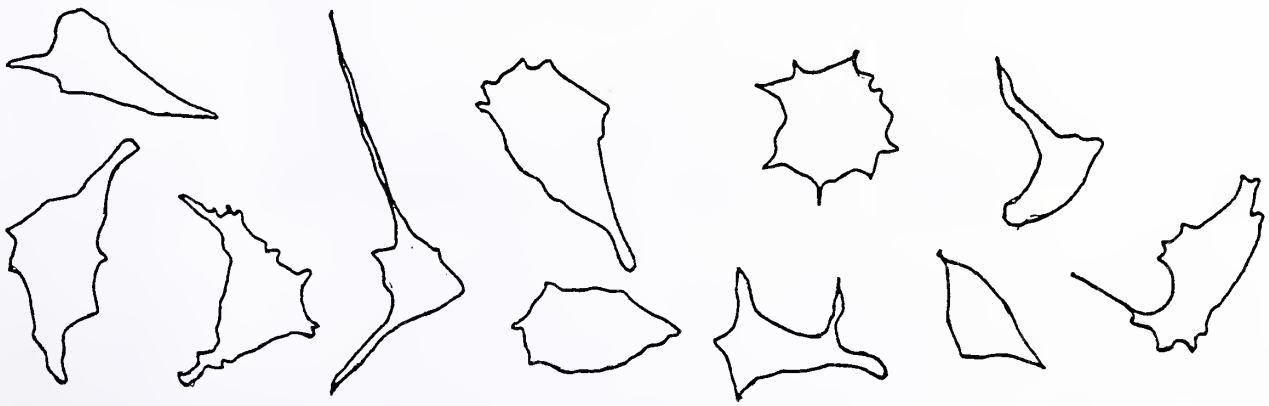
Figure 12. Serial tracings of cells incubating in 100 ng/ml NGF



Day 1, 5 culture days



Day 3, 7 culture days



Day 5, 9 culture days

Figure. 13, 14, 15. Serial tracings of cells incubating in DBcAMP-Theophylline.

A. Day 0 , 3 culture days

B. Day 3, 6 culture days

C. Day 5, 8 culture days

D. Day 6, 9 culture days

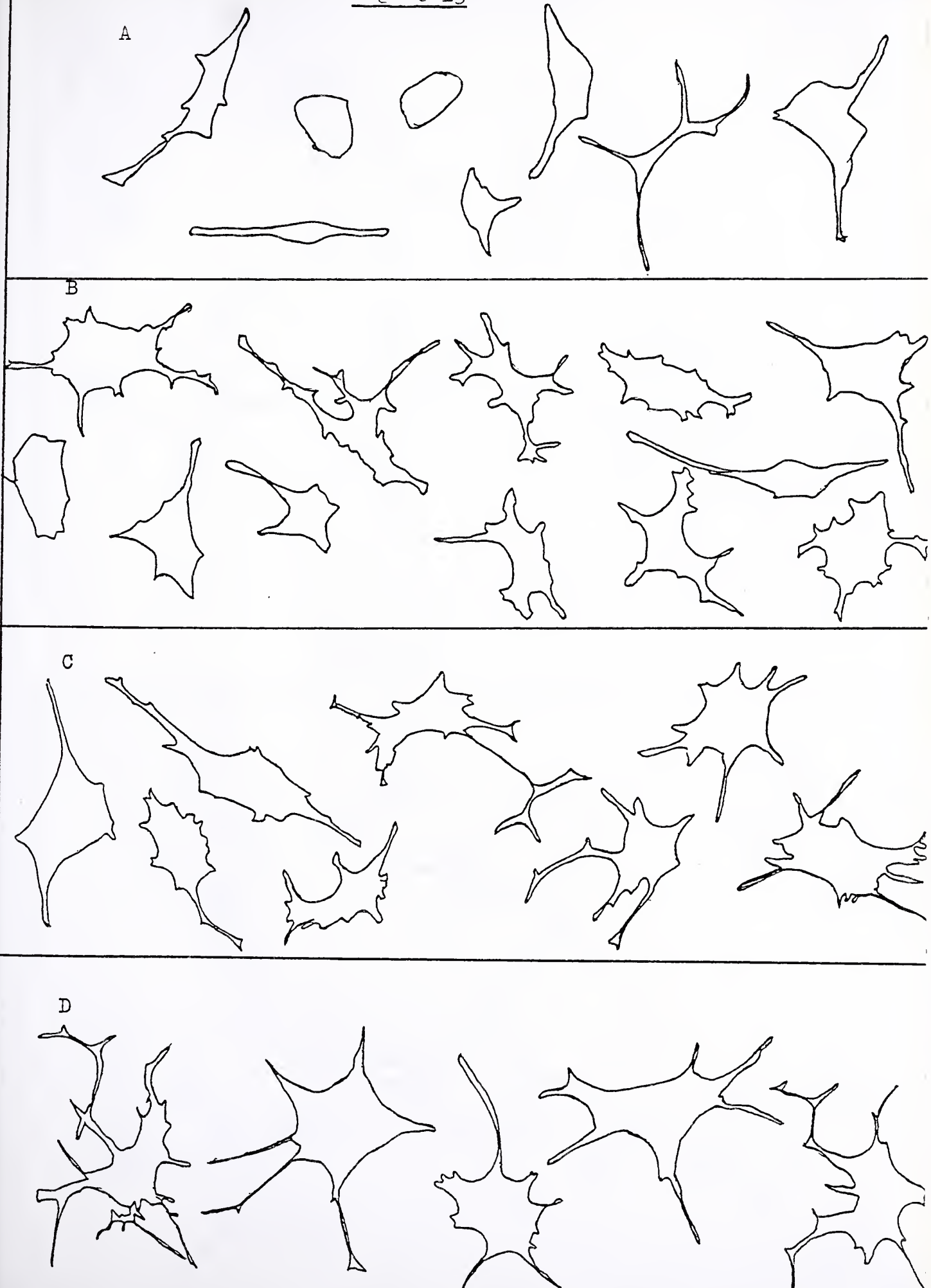
E. Day 8, 11 culture days

F. Day 11, 14 culture days

G. Day 14, 17 culture days

H. Same culture after changing the media back to MEM with 30% fetal calf serum, for 2 days.

Figure 13



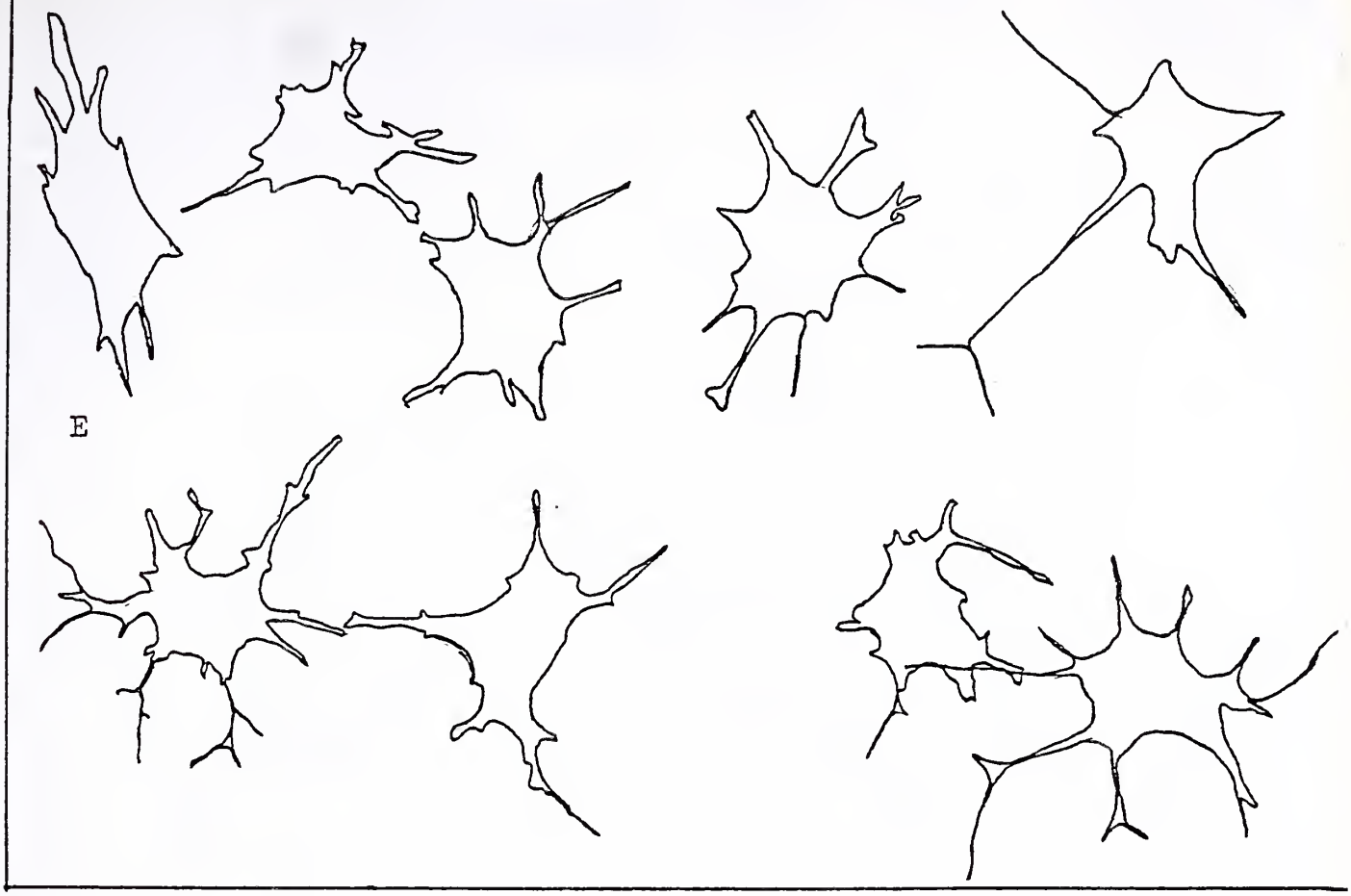


Figure 14

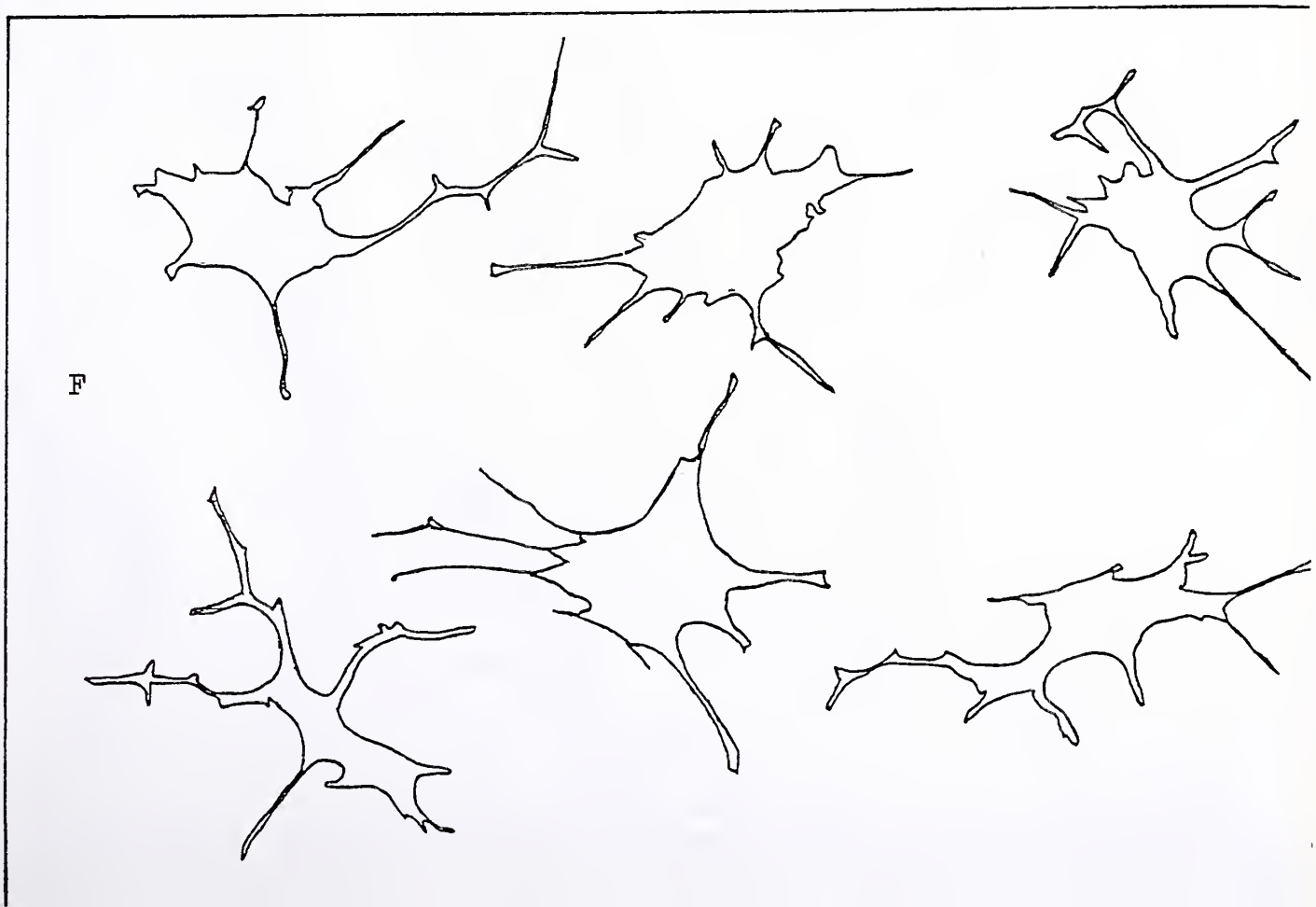
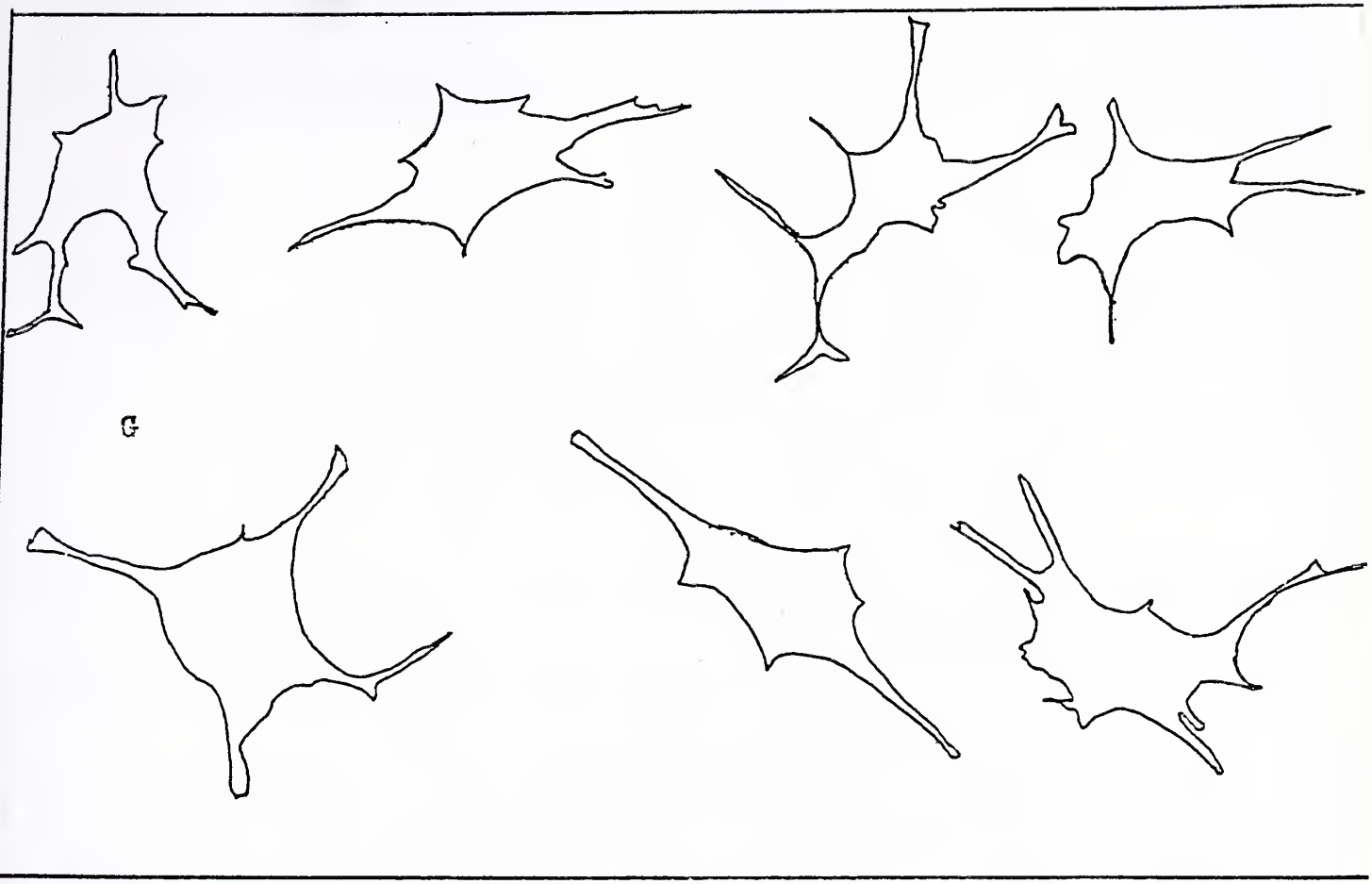


Figure 15



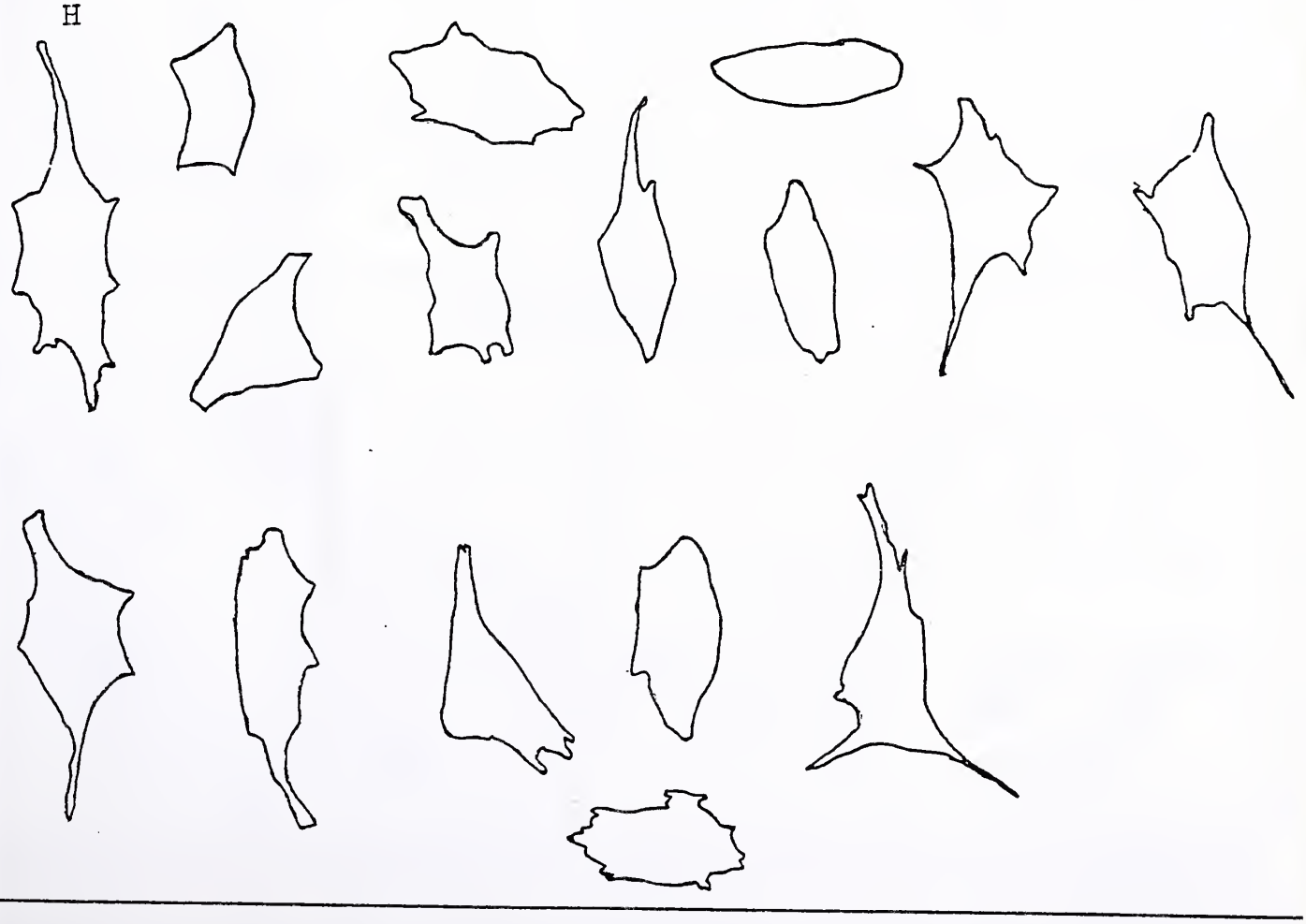
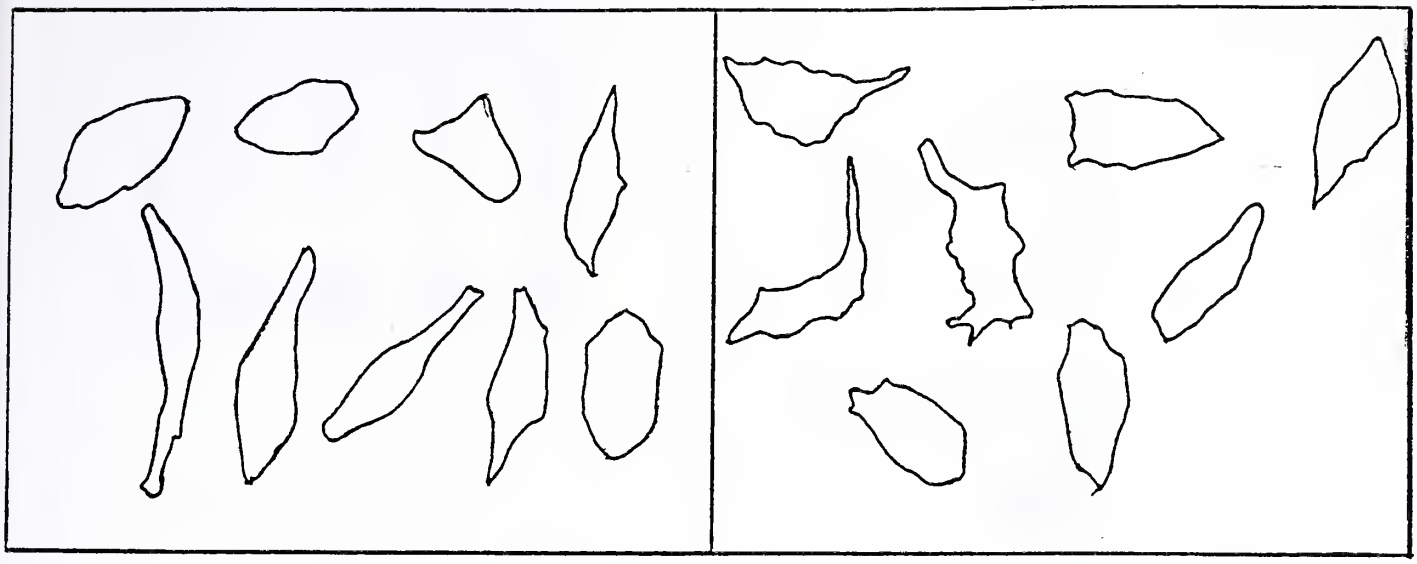


Figure 16. Tracings of cells at Day 5.

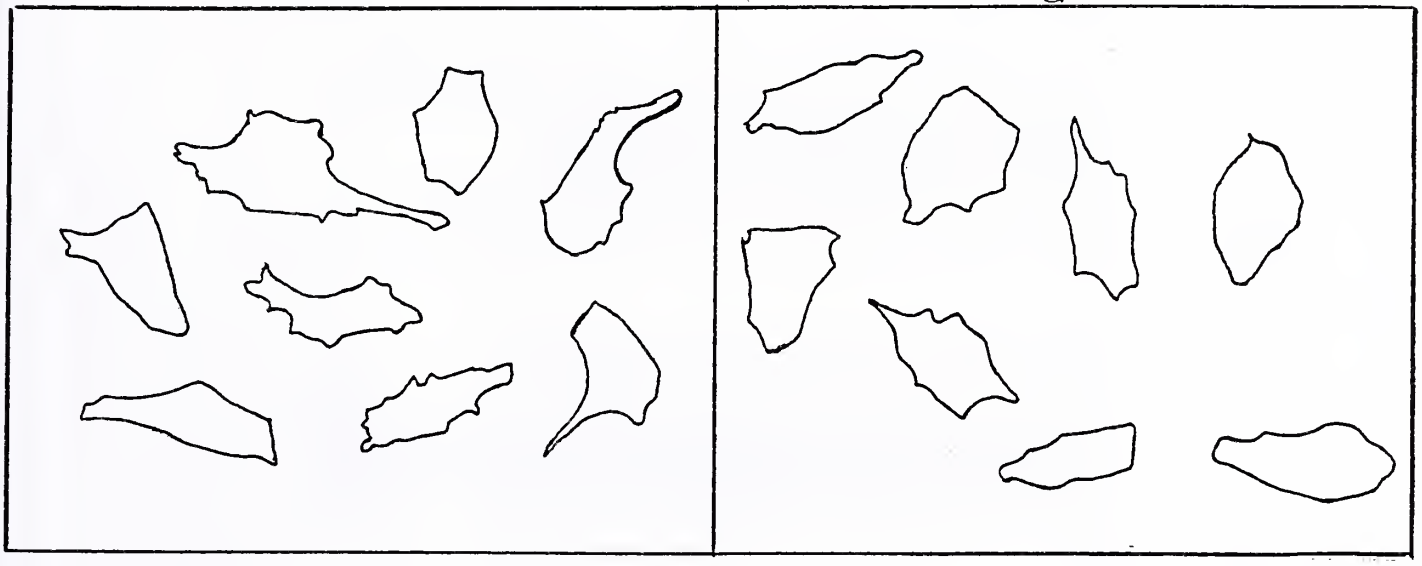
CONTROL

10 ng/ml

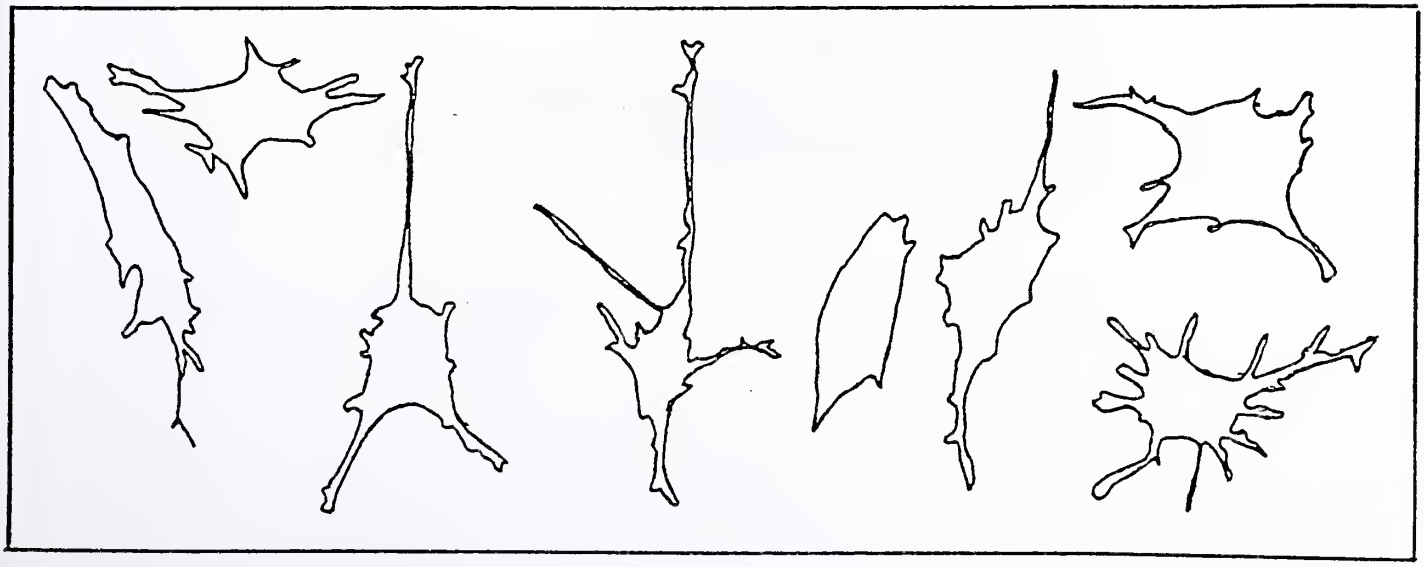


50 ng/ml

100 ng/ml



DBcAMP-Theophylline



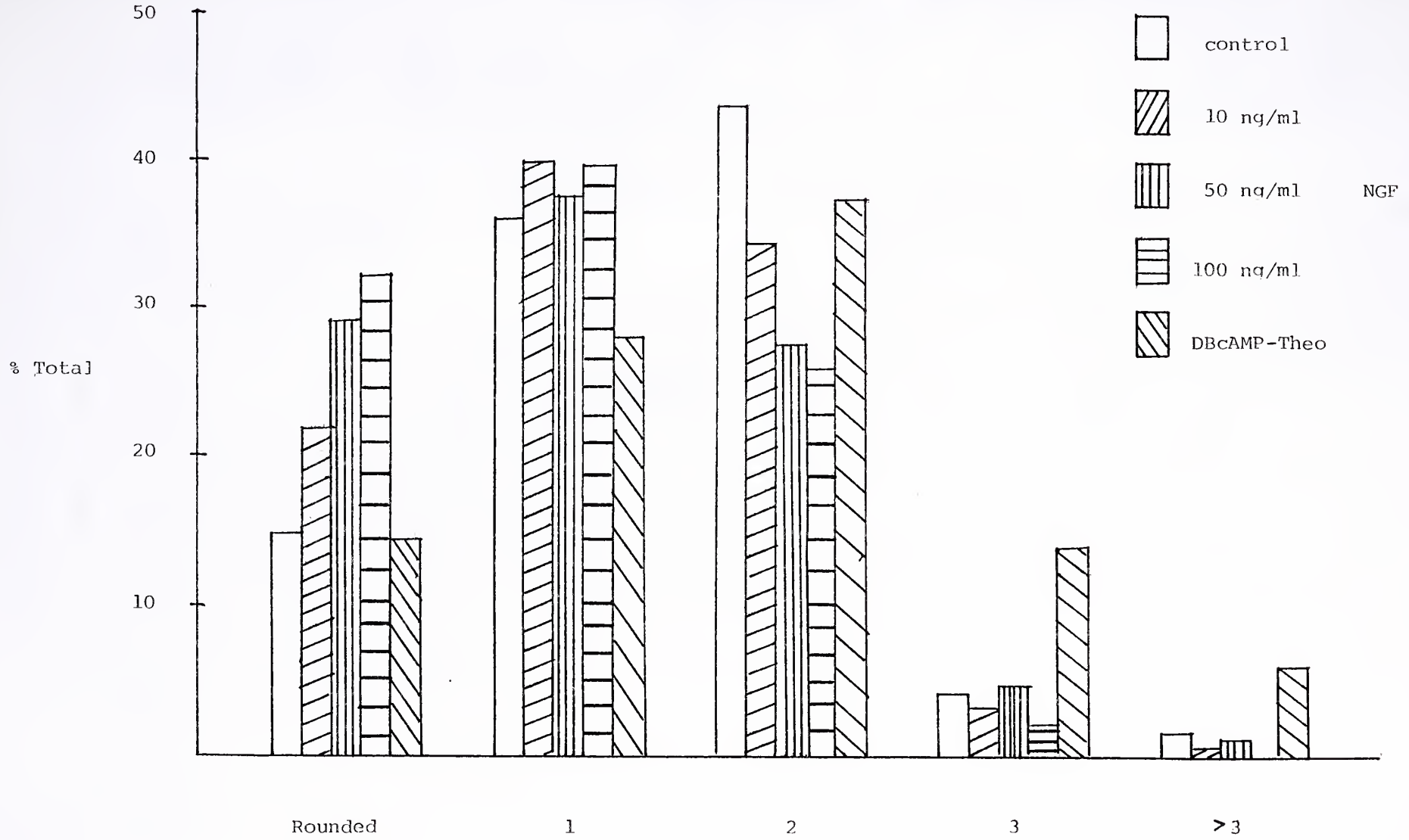


Figure 17: Distribution of melanocytes according to number of dendrites

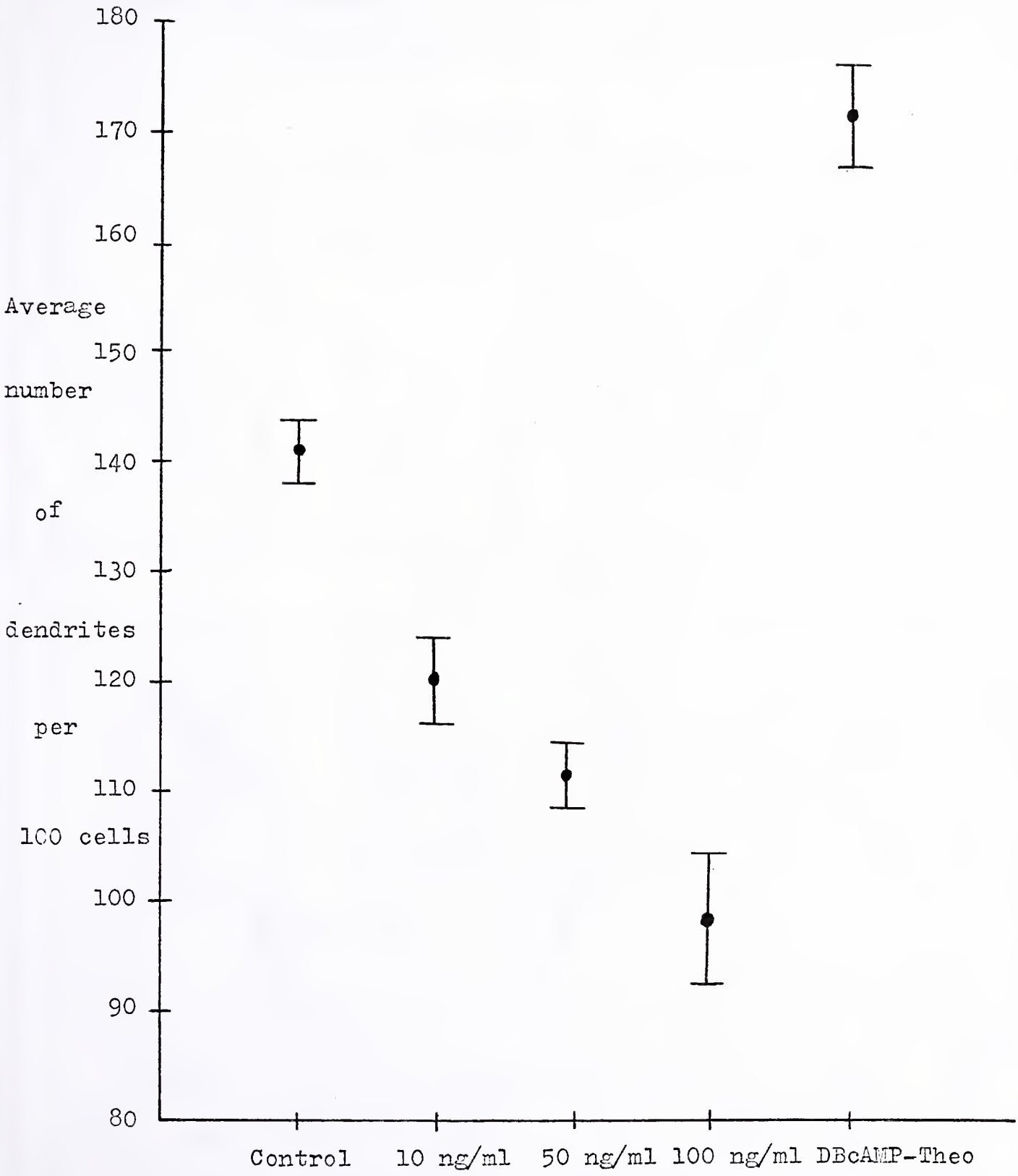
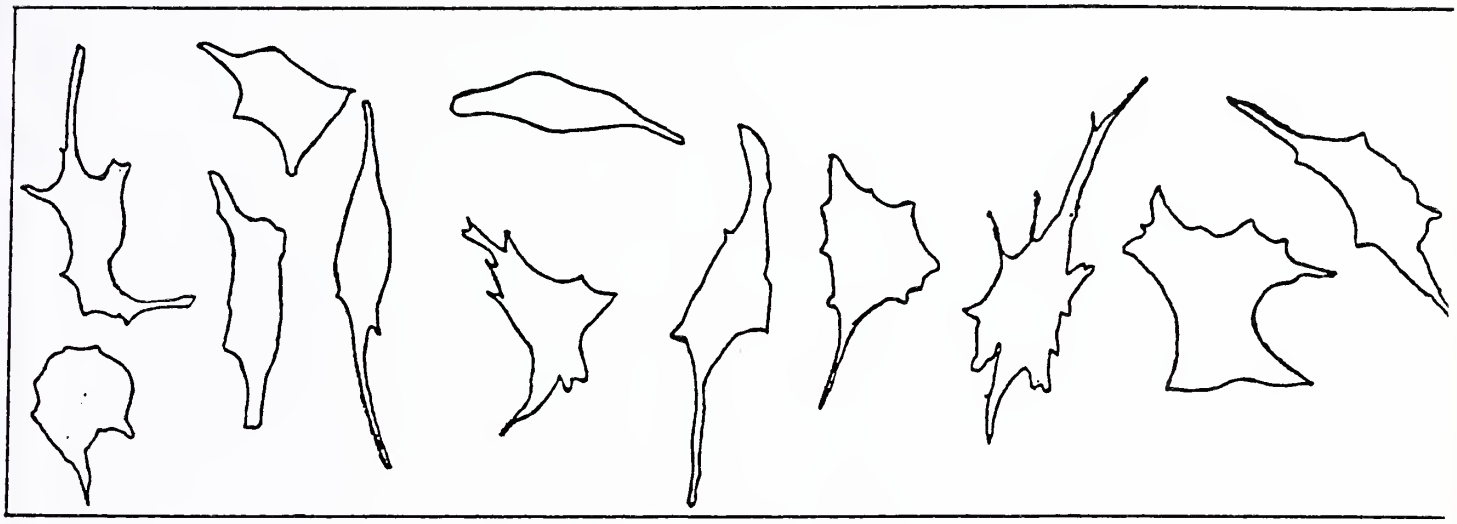


Figure 18: The average number of dendrites/100 cells in all groups from Day 5.

Figure 19. Tracings of cells after the media was changed from 50 ng/ml NGF to DBcAMP-Theophylline.

50 ng/ml NGF



DBcAMP-Theophylline

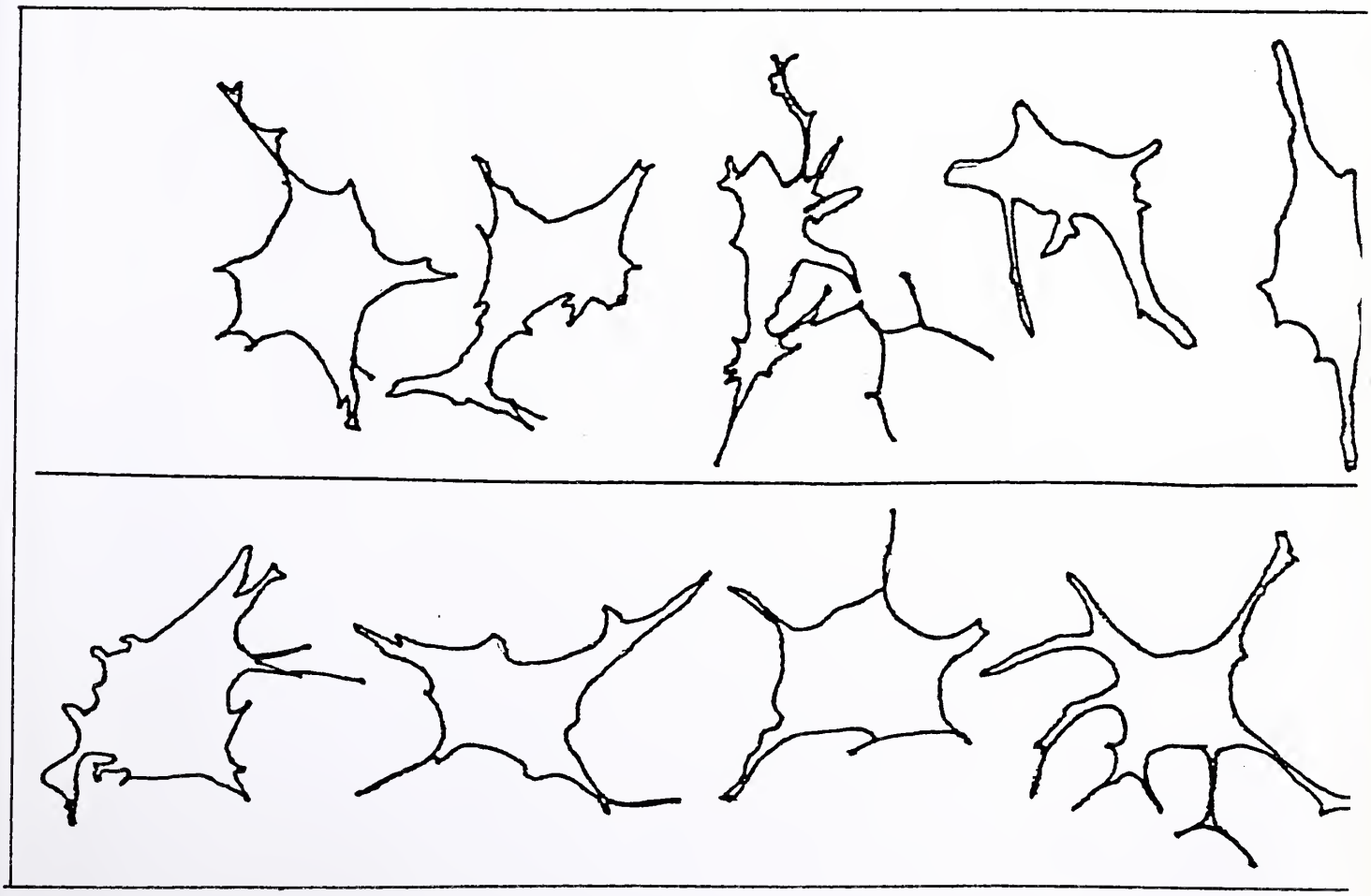
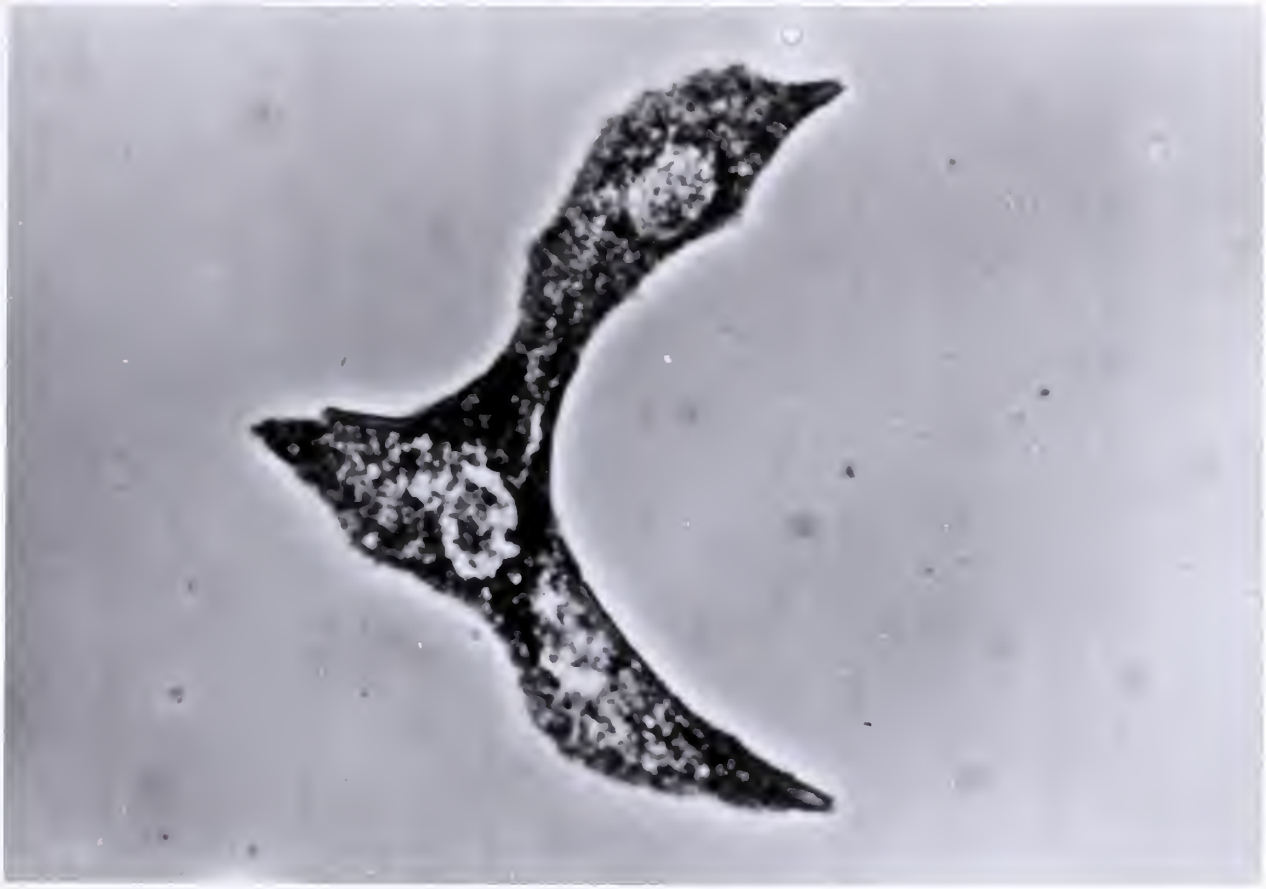


Figure 20. CONTROL: 3 melanocytes in contact with each other. This could represent cell divisions with daughter cells overlapping each other due to low motility rate. 400 X.

Figure 21. CONTROL: a dendritic melanocyte in contact with several keratinocytes. Pigment granules can be seen within the cytoplasm of the keratinocytes. 400 X.



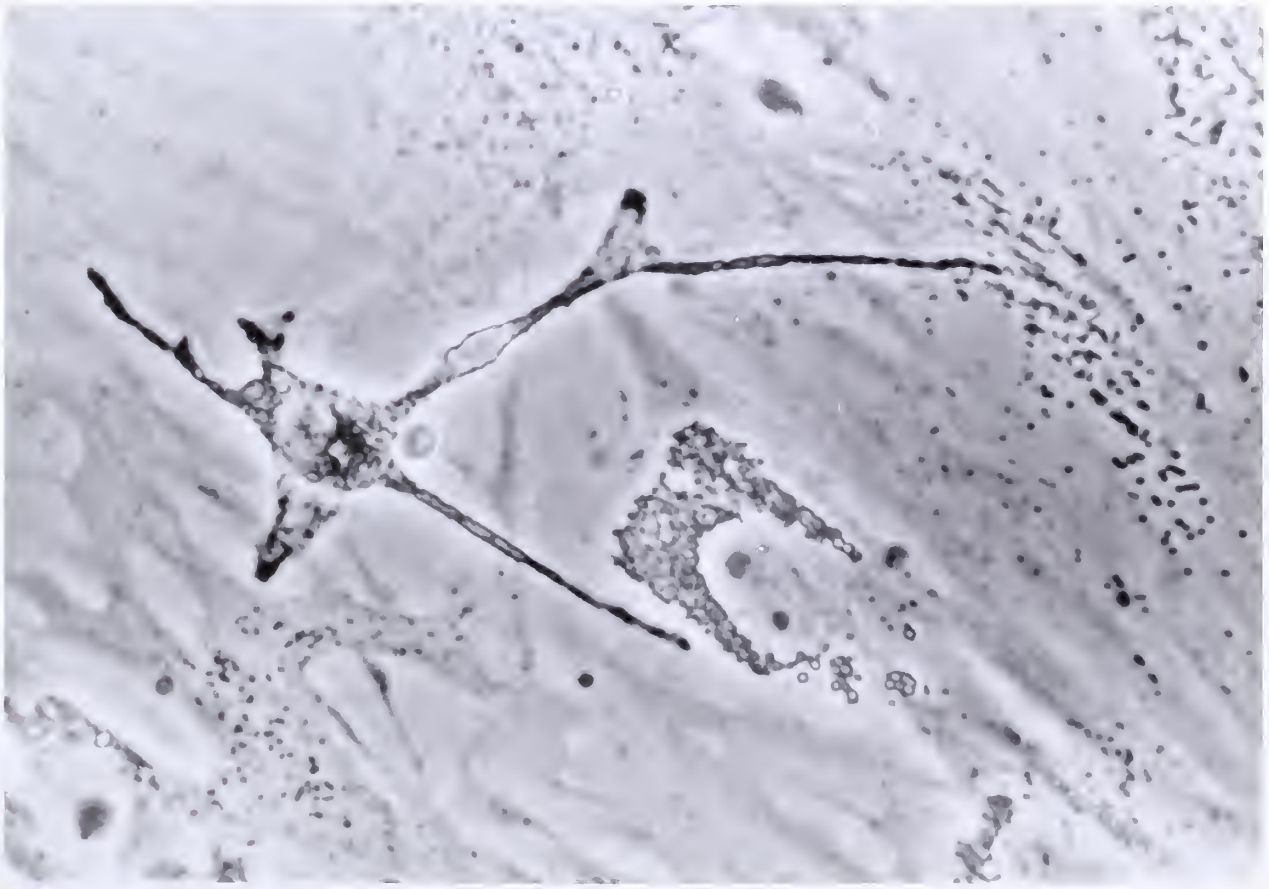
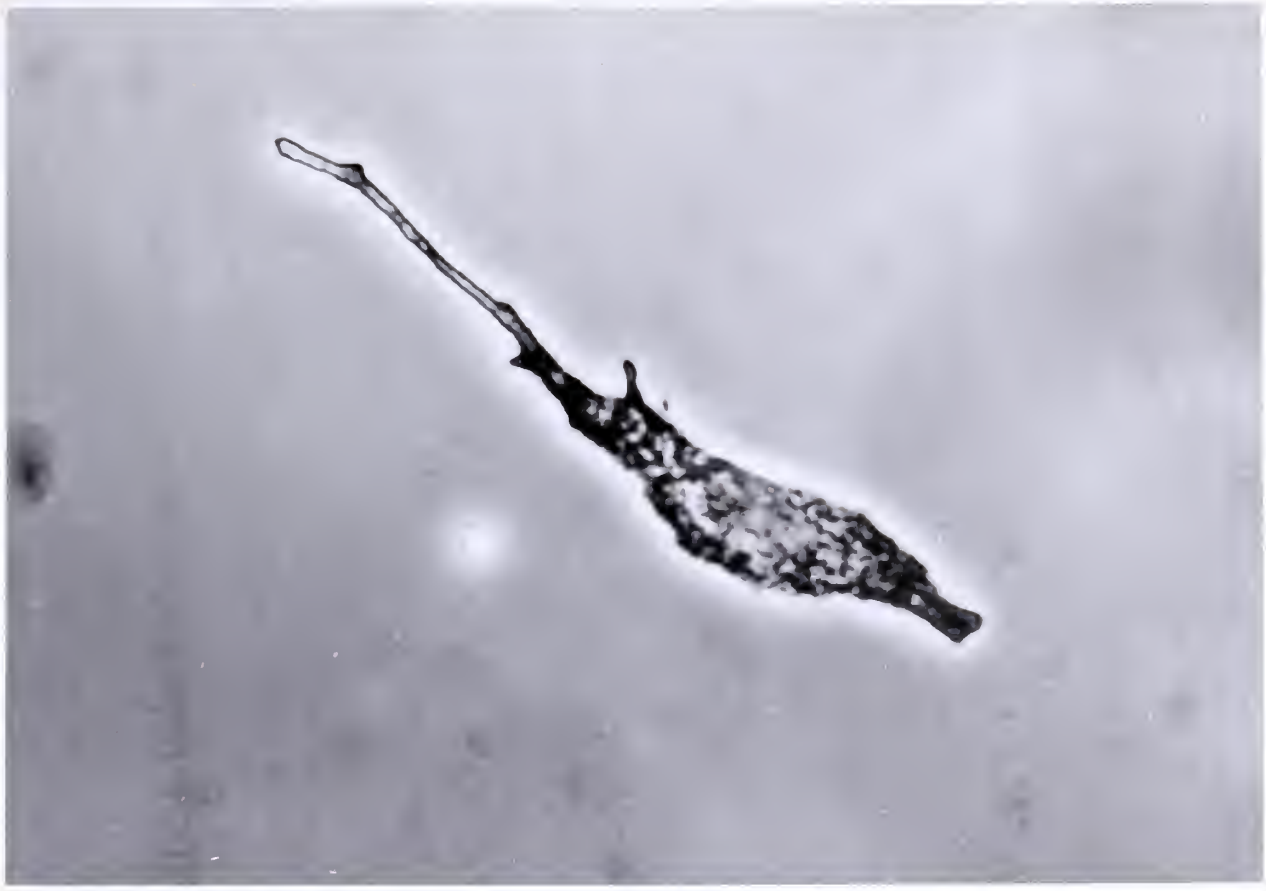


Figure 22. 10 ng/ml NGF : a bipolar melanocyte with simple dendrites.
400 X.

Figure 23. 10 ng/ml NGF: a cluster of melanocytes. It appears that NGF does not inhibit cell division.
160 X.



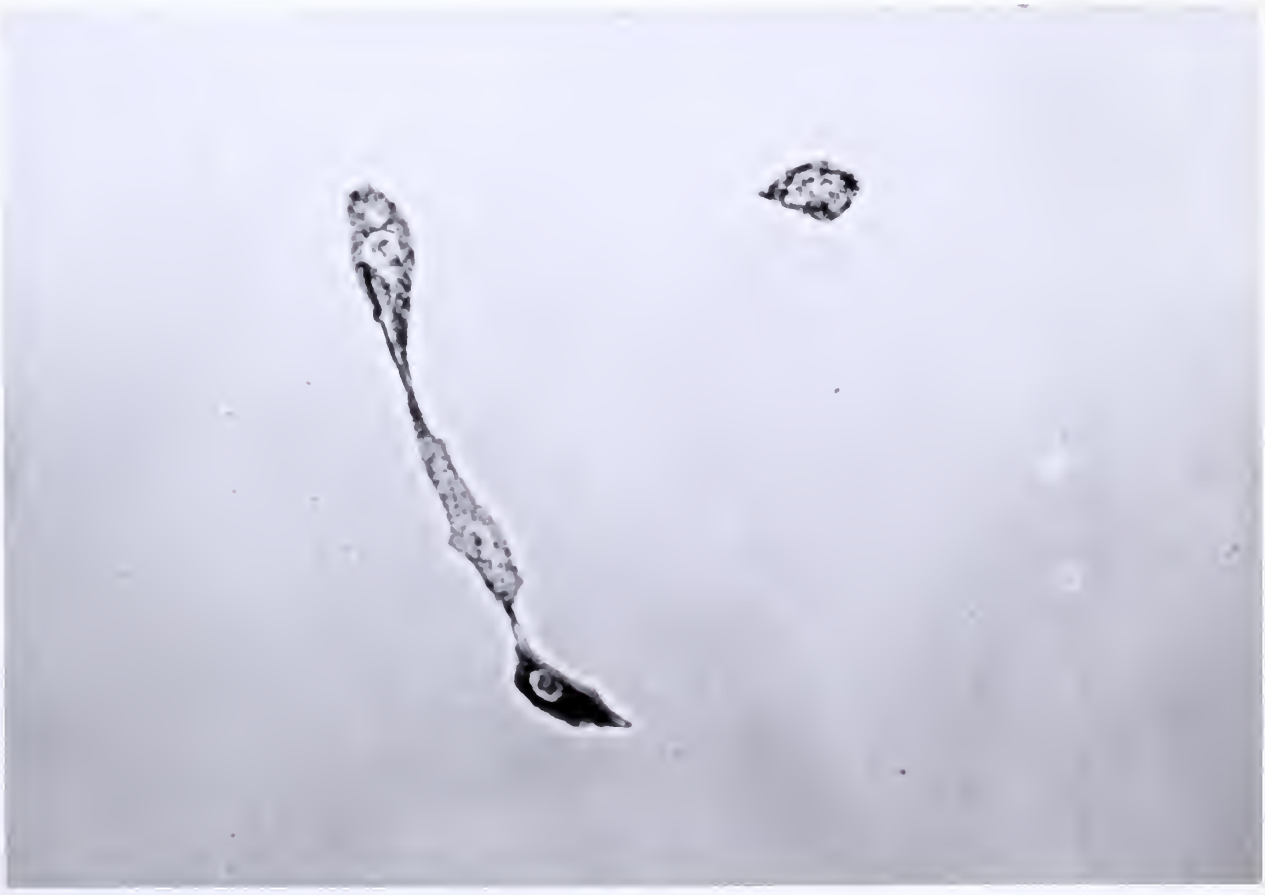
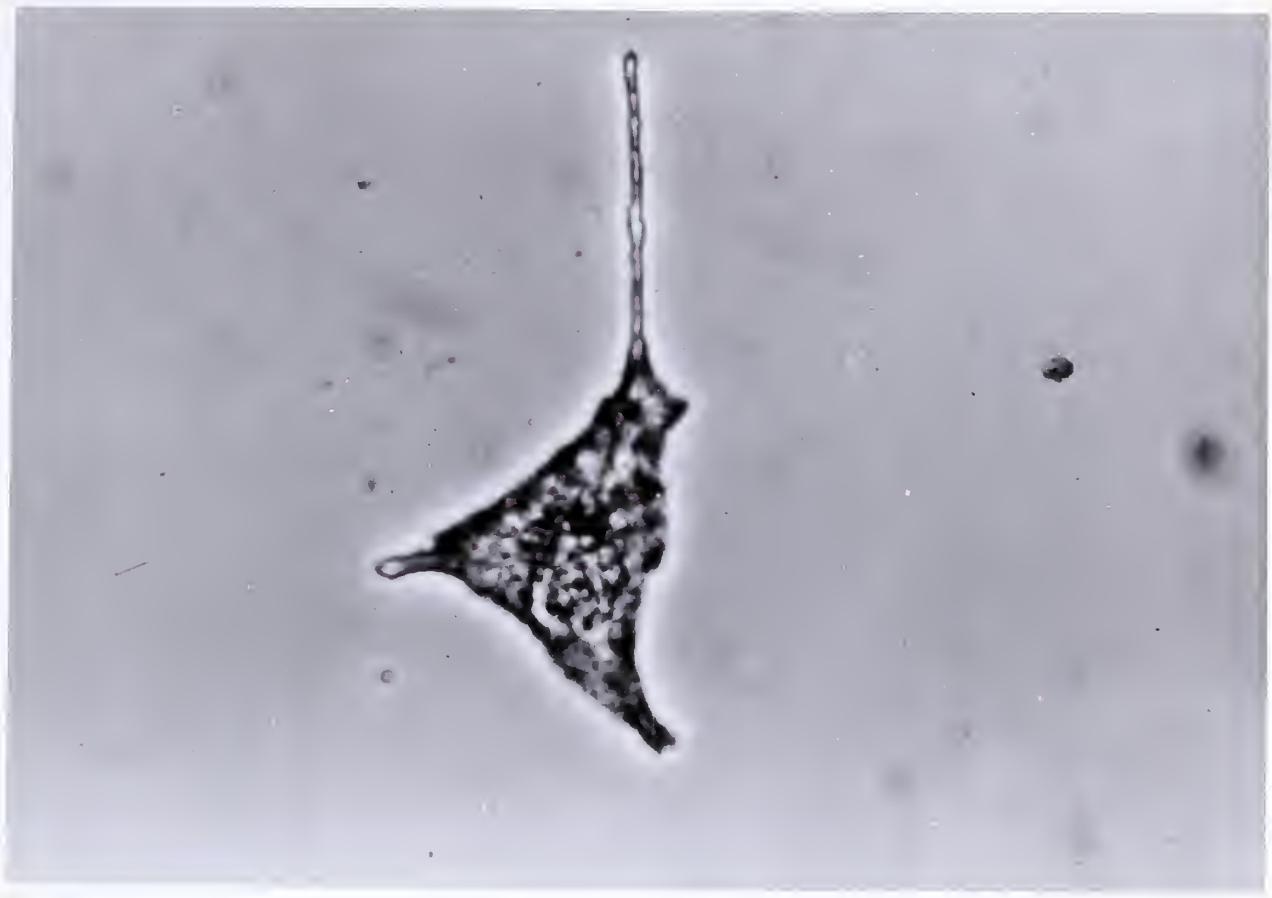


Figure 24. A melanocyte prior to the addition of 50 ng/ml NGF.
400 X.

Figure 25. 50 ng/ml NGF: same melanocyte as fig.24 20 minutes after exposing to NGF. No marked difference in shape or distribution of the melanin granules could be detected.
400 X.



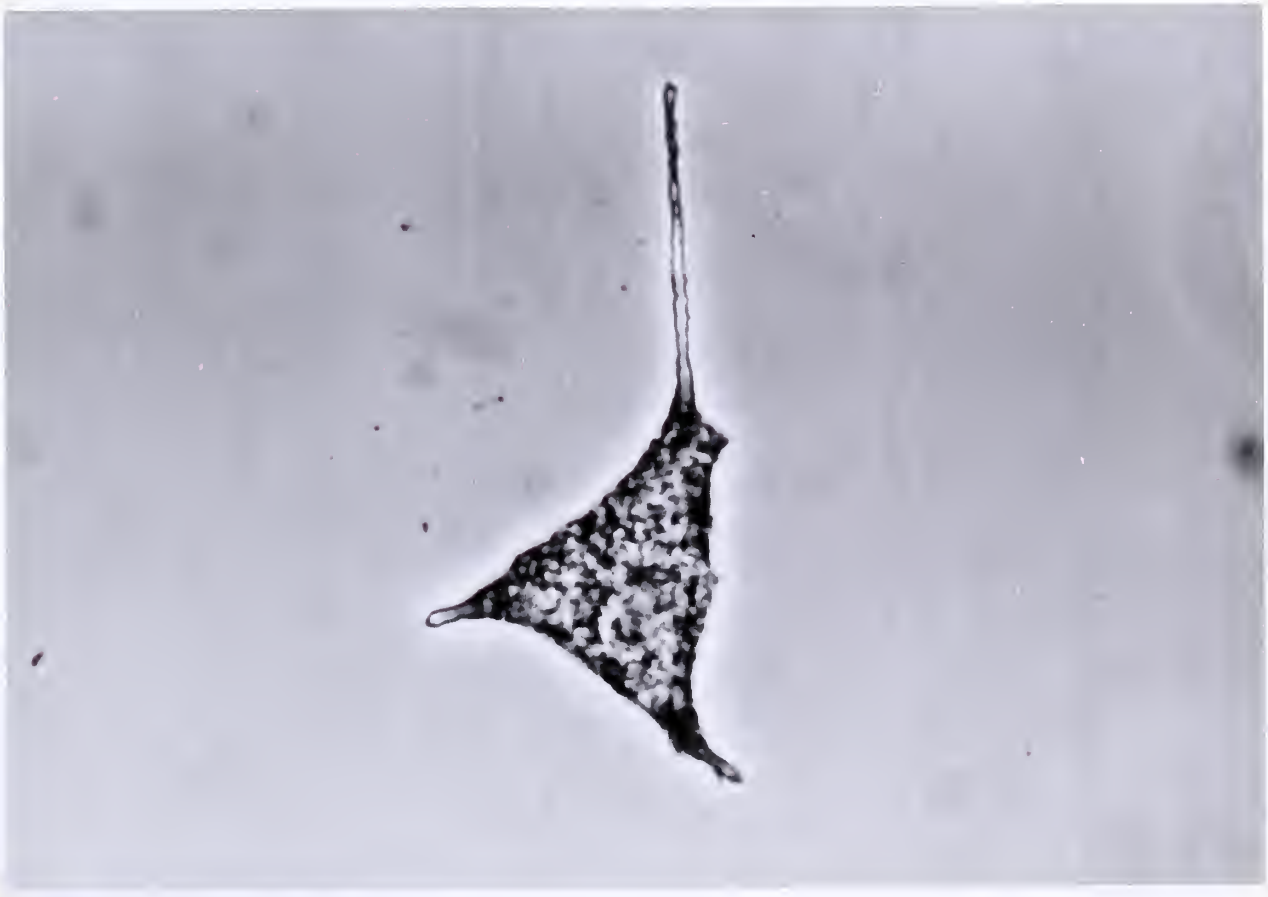
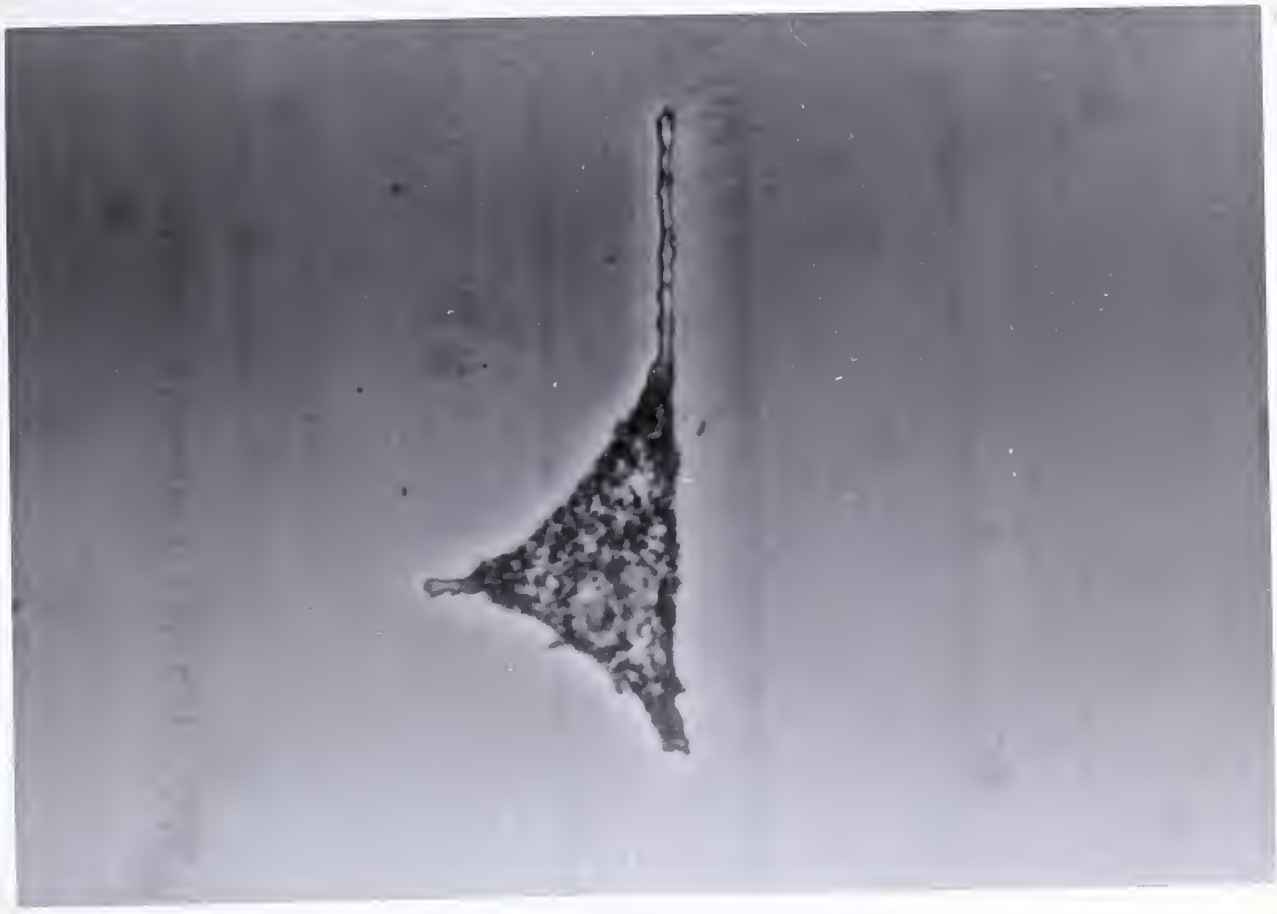


Figure 26. 50 ng/ml NGF: the same cell after incubating in NGF for 6 hours. No significant difference could be detected, as compared to figure 24. 400 X.

Figure 27. 50 ng/ml NGF: 2 hours after NGF was added. Note the "ruffling" of the cell membrane. These cellular processes did not lengthen after several hours of observation. This effect appeared to be transient and the membrane resumed its smooth contour within 4-6 hours.
400 X.



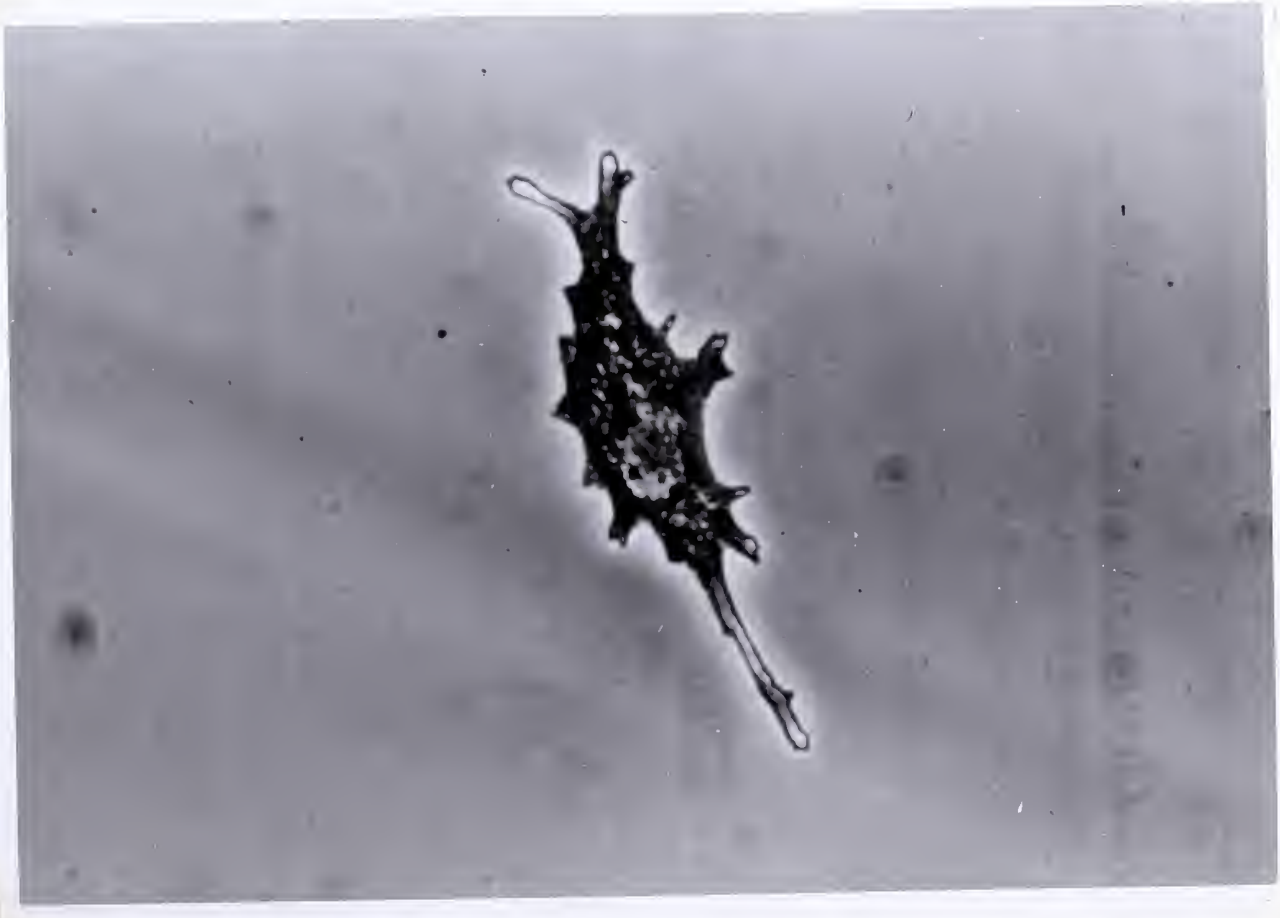
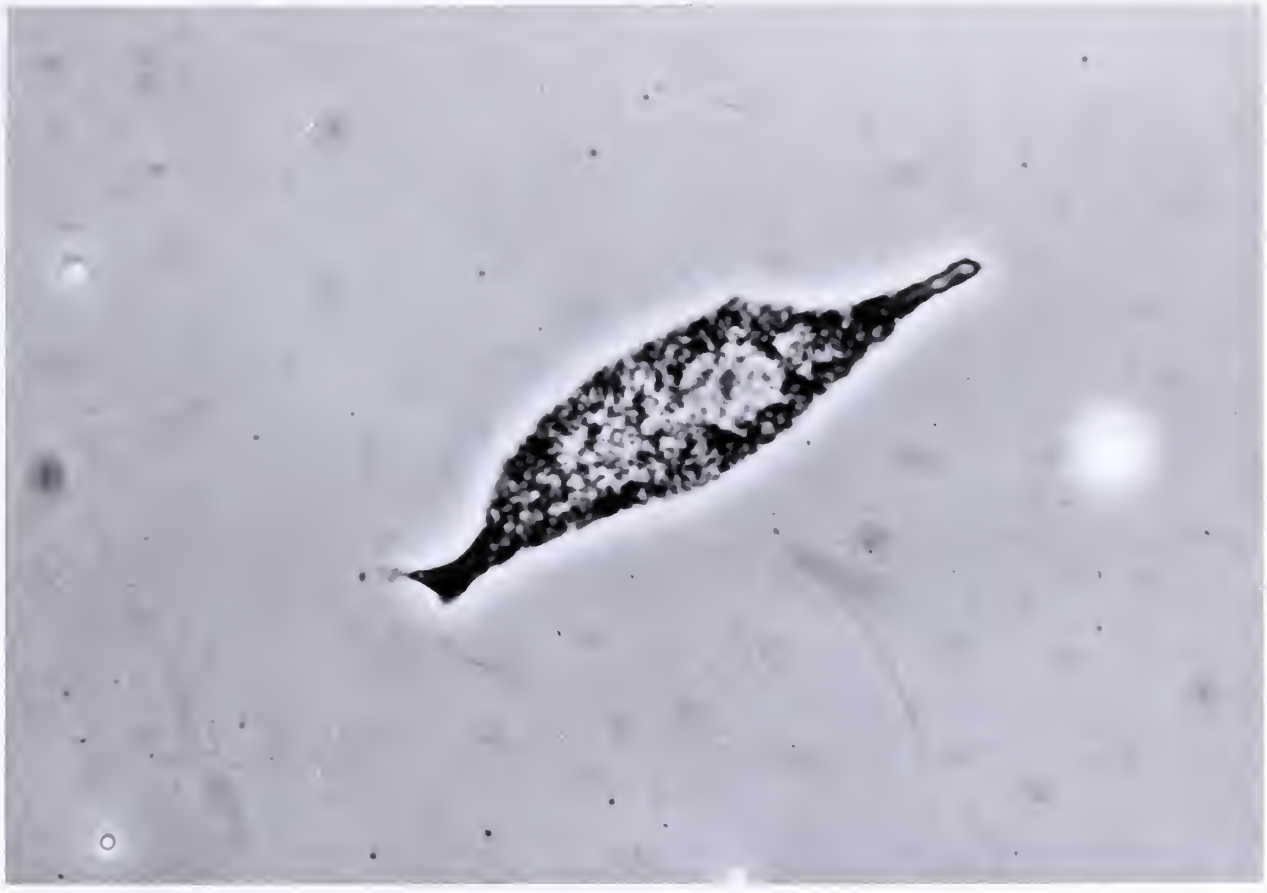


Figure 28. 100 ng/ml NGF: a typical melanocyte in this group with simple dendrites.
400 X.

Figure 29. 100 ng/ml NGF: 2 melanocytes in contact with each other. NGF at this concentration does not appear to inhibit, nor does it stimulate melanocyte growth.
400 X.



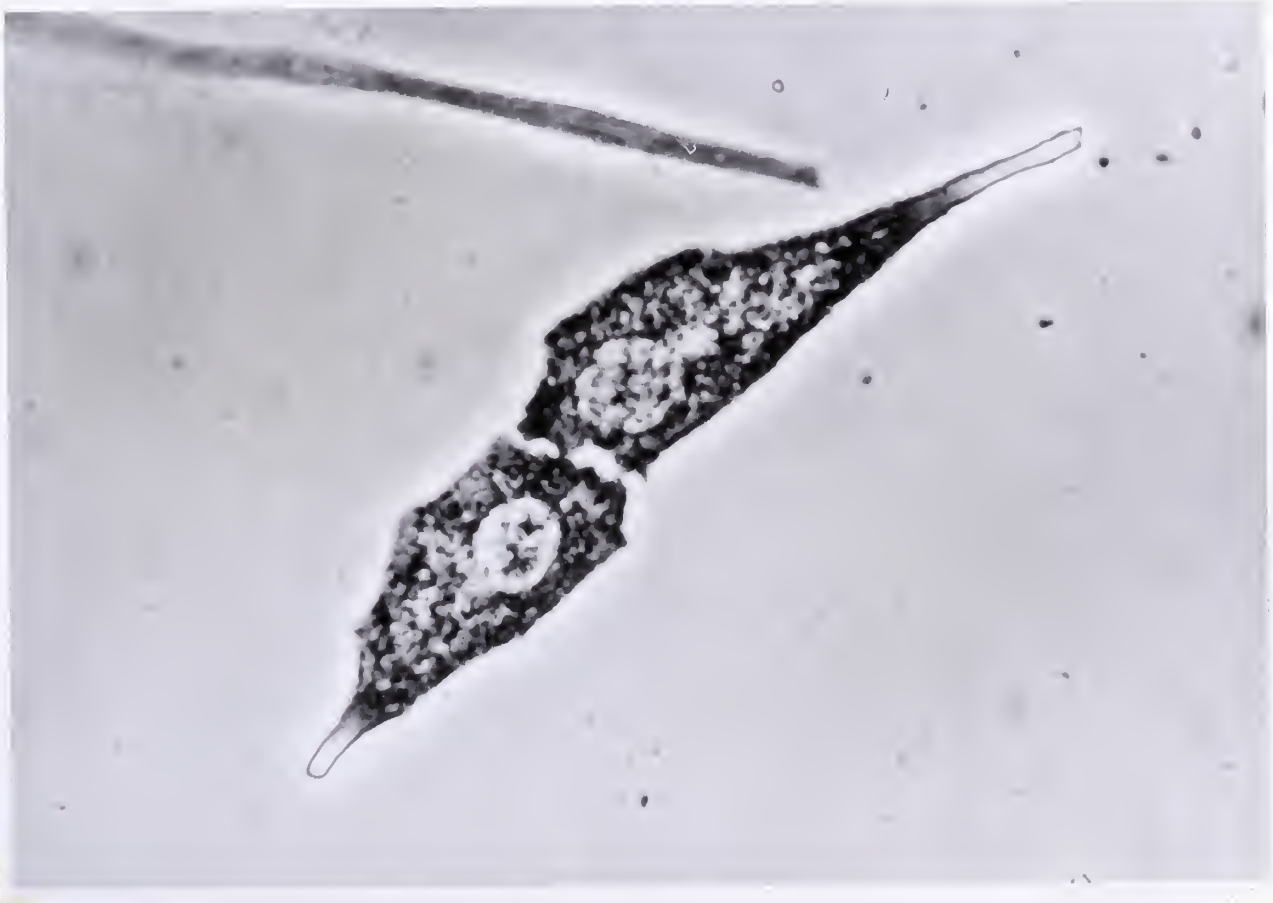
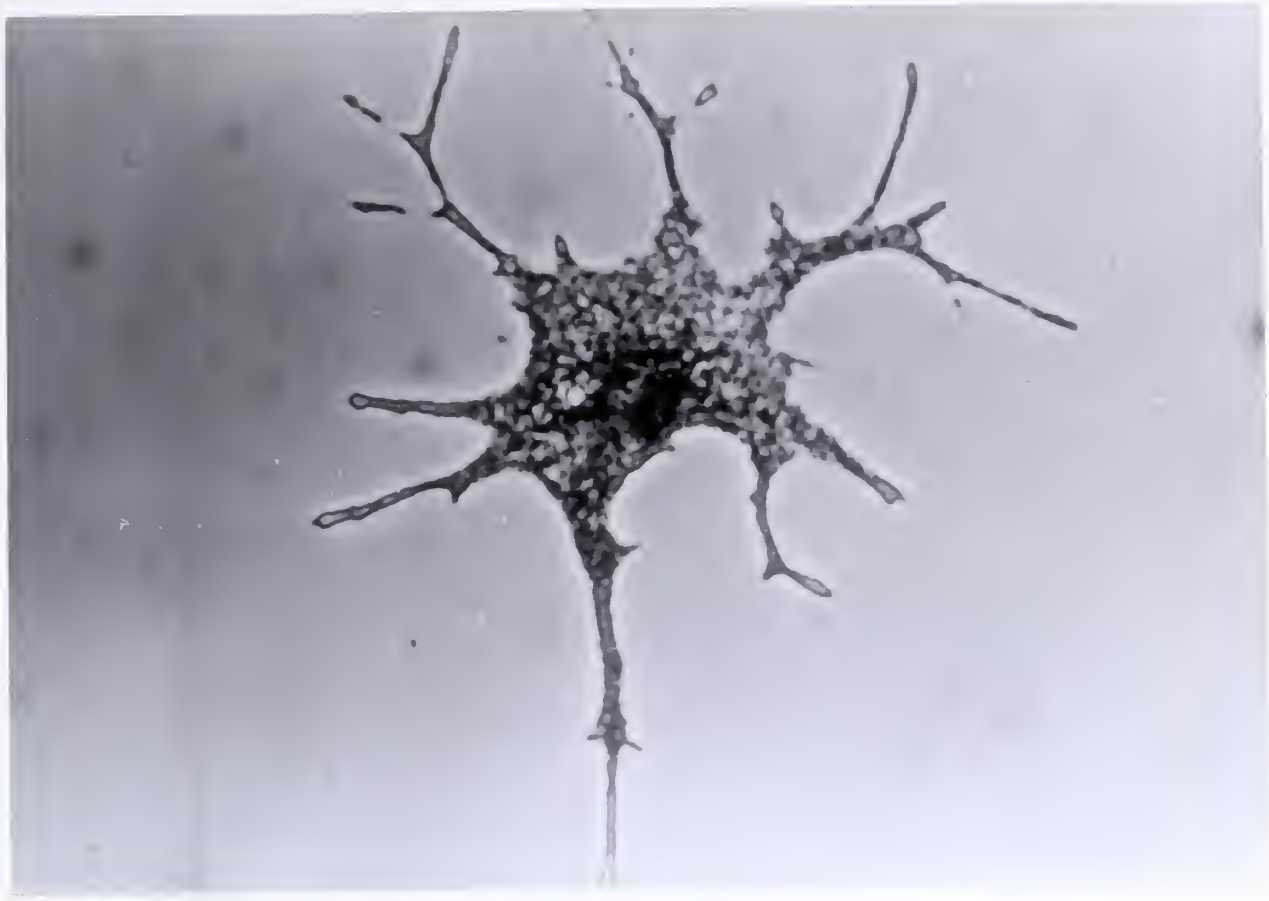


Figure 30. DBcAMP-Theo: melanocyte seen after 5 days of exposure to DBcAMP-Theo. Note the increase in number of dendrites, with secondary branchings. Fragmentation of dendrites could be seen without detrimental effect to the melanocyte. 400 X.

Figure 31. DBcAMP-Theo: another extremely dendritic melanocytes after 5 days of exposure to DBcAMP-Theo. Note the compexity of the dendrites with secondary and tertiary branchings. 400 X.



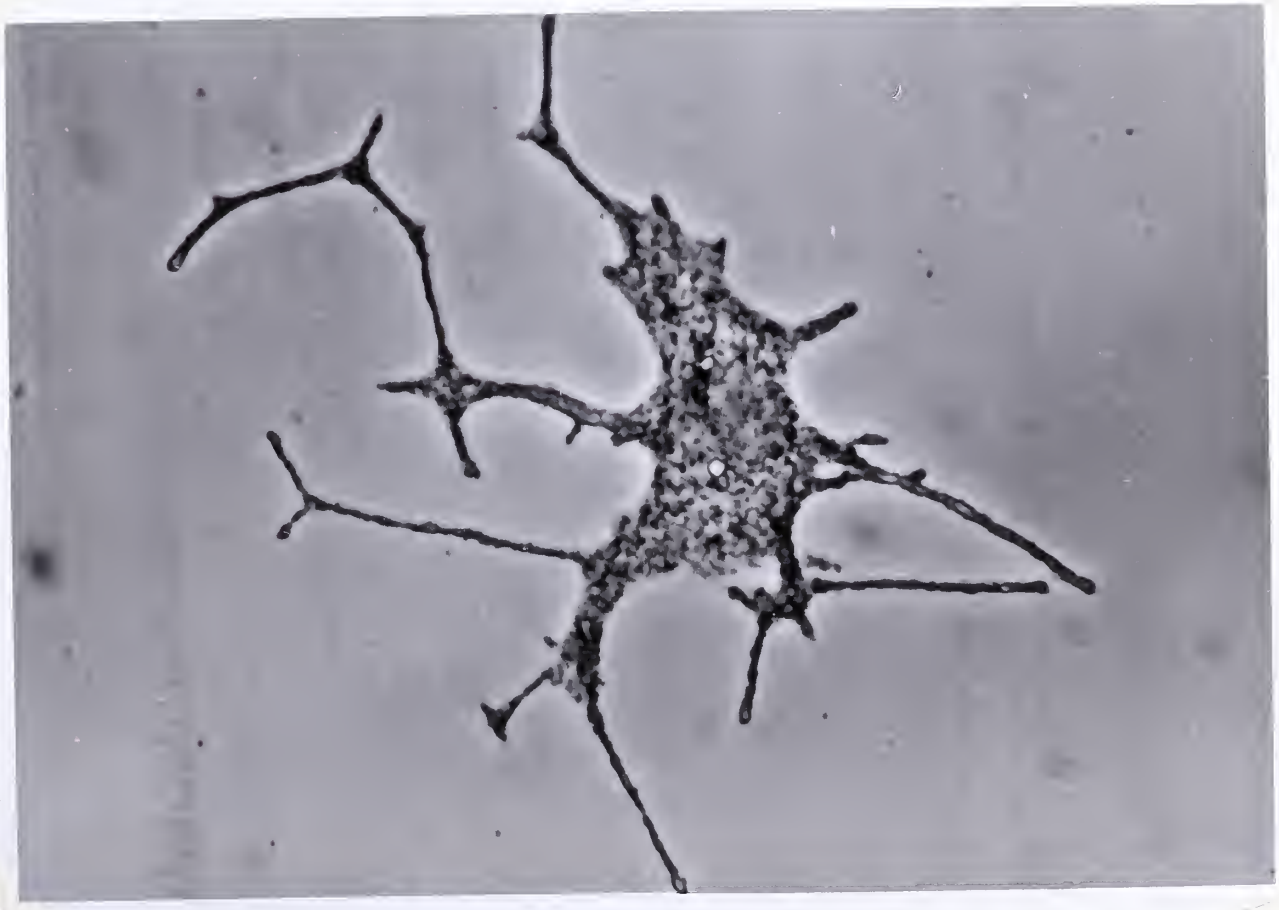
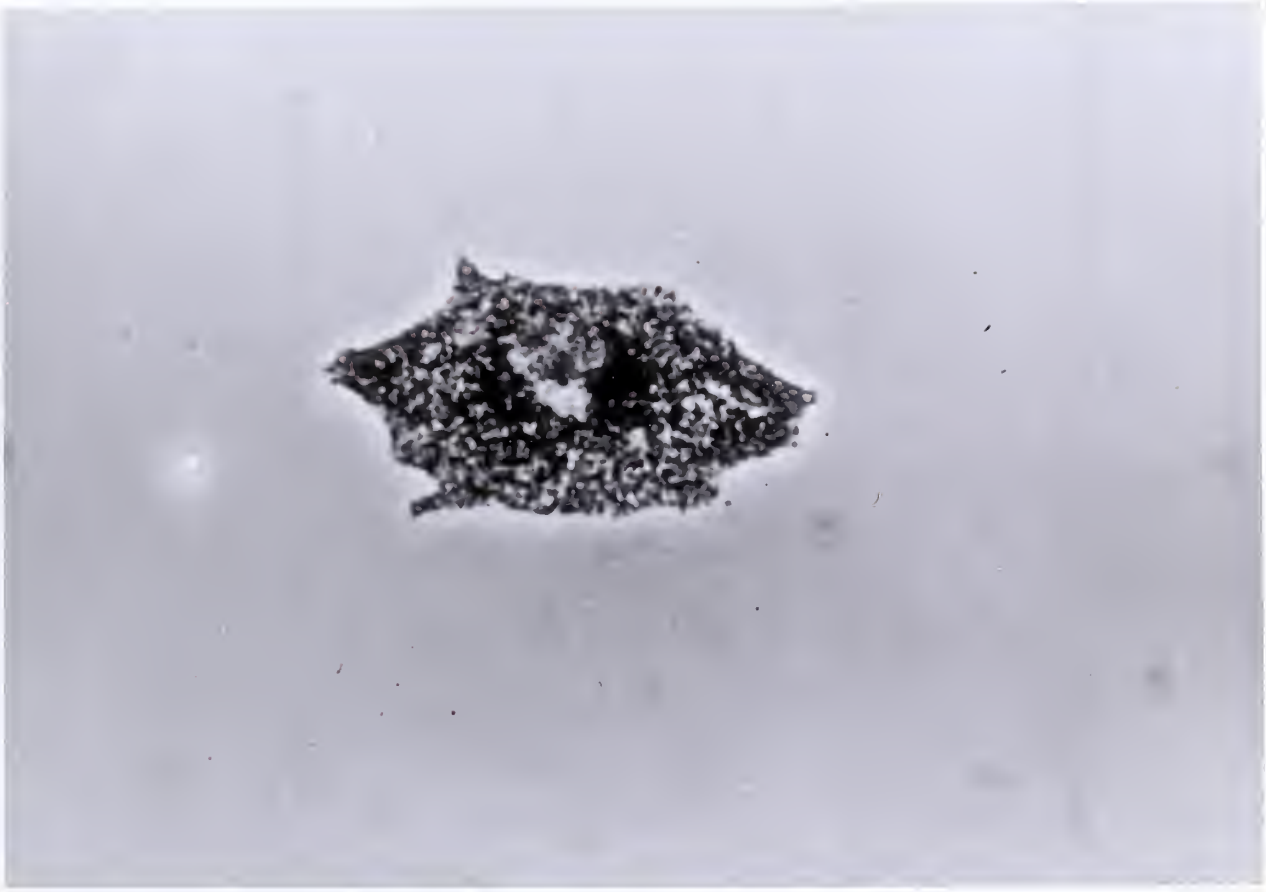


Figure 32. This is the same melanocyte in figure 31, 2 days after the culture medium had been change back to Eagle MEM with 30% fetal calf serum. Note the marked decrease in the number of dendrites as compared to figure 31.

400X.



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-R.C.B. for her emotional support.

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